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STUDIES WITH *BACILLUS POLYMYXA*

IV. NITROGEN REQUIREMENTS IN RELATION TO 2,3-BUTANEDIOL PRODUCTION FROM STARCH[1]

BY H. KATZNELSON[2]

Abstract

Yeast extract, casein hydrolysate, and a mixture of 13 to 20 amino acids were found to be superior to simpler substances such as ammonium sulphate, urea, potassium nitrate, or asparagine as sources of nitrogen for *Bacillus polymyxa* in relation to production of 2,3-butanediol from starch. The complex sources of nitrogen were more or less interchangeable for most, but not all, strains of this organism with regard to effectiveness for diol production, but the addition of yeast extract to either casein hydrolysate or amino acids resulted in a slightly higher yield of diol and an increase in the diol/ethanol ratio from 2 to 2.6 or higher.

The requirements of different strains for specific amino acids varied somewhat, but the need for isoleucine and asparagine was common to the four strains studied. A fairly good fermentation was obtained with the most efficient of these strains in a medium containing isoleucine, tyrosine, glycine, methionine, and asparagine after three days' incubation. The fermentation went to completion after five days with certain concentrations of these five acids, an effect that was achieved in three days by the addition to these of eight other acids but not by the addition of ammonium sulphate. Suppression of diol production by omission of certain of these amino acids was marked at three days but was largely overcome at five. Cystine (in concentrations above 0.0125%) was inhibitory to the four strains studied and phenylalanine (0.02%) to one, after three but not after five days.

Introduction

In the preceding paper of this series (8) it was shown that *Bacillus polymyxa* could grow in a medium containing casein hydrolysate as the nitrogen source and that this complex could be replaced by a mixture of 18 amino acids, urea, or ammonium sulphate. Yeast extract was found to be superior to all these substances and has also been shown to exert a favourable effect on production of 2,3-butanediol from wheat (7, 9). Subsequent to the publication of the above work (8) growth studies were continued with other strains of *B. polymyxa;* it soon became apparent that for most of these, casein hydrolysate, a mixture of amino acids (slightly different from that used in the earlier investigation), or yeast extract was far superior to simpler compounds such as urea or ammonium sulphate. Growth with amino acids was usually equal, and often superior, to that with casein hydrolysate, but yeast extract was always the most effective substance. However, attempts to

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replace the latter with 15 growth-promoting substances (vitamin B complex, purine, and pyrimidine bases) in a medium containing casein hydrolysate or amino acids as a source of nitrogen were unsuccessful. Further studies were made to determine which of the amino acids used were essential for growth of this organism. The omission of leucine or isoleucine from a mixture of 18 acids retarded growth appreciably at 48 hr. but this effect was not evident after 96 hr.; none of the remaining acids appeared to exert any specific effect.

Similar work was carried out on the influence of various nitrogenous substances on production of 2,3-butanediol rather than on growth. Starch was used as the carbon source instead of glucose since the latter was found to be toxic to *B. polymyxa* in the concentrations required to give appreciable yields of the diol. The proposed utilization of starch as the substrate for production of butanediol (4, 9, 17), owing to difficulties encountered in the use of whole wheat, heightened the interest in this investigation, the results of which are reported in this paper.

Experimental

A basal medium containing, per litre, 0.3 γ biotin and 5 ml. each of inorganic solutions *A* and *B* of Snell and Strong (15) was prepared in double strength and dispensed in 50 ml. amounts in 500 ml. Erlenmeyer flasks. The ingredients to be tested were then added and the volume of the solution brought to 100 ml., after which the reaction was adjusted to pH 6.8 and 4 gm. of Stein-Hall wheat starch added. The medium was sterilized for 20 min. at 15 lb. pressure and after cooling received 1 gm. calcium carbonate (sterilized separately in tubes). Inoculum was prepared by centrifuging a 24-hr. culture (in casein-hydrolysate–biotin–glucose medium) and resuspending the cells in 10 ml. sterile distilled water, 1 ml. being used per flask. After an incubation period of 72 or 120 hr. at 28° C. the contents of the flasks were filtered and the filtrates analysed for 2,3-butanediol (9). Since changes in diol production were usually accompanied by similar changes in production of ethyl alcohol (diol/ethanol ratio being about 2), analyses for the diol alone were usually made. Where the results suggested a possible variation of this ratio, ethanol was also determined. The data given in the tables are representative of results obtained in frequently repeated experiments. All glassware was carefully cleaned (with acid dichromate) and rinsed.

A 4% starch suspension was found to be the most convenient to work with as a greater concentration cooled to a gelatinous mass that was difficult to manipulate. Malting would have permitted use of higher concentrations of starch but this was of course impossible in these studies in view of the necessity of avoiding introduction of unknown organic substances (other than those present in the starch itself). According to the calculations of Ledingham *et al.* (9), a theoretical yield of diol and ethanol (designated "total products") of 2.2% may be expected from 4% starch, although they state that this may never be obtained even under optimum conditions. However, 85 to 95% of this theoretical yield was usually obtained with the most efficient strain in the collection (No. 47).

Experimental Results

Influence of Different Sources of Nitrogen

A preliminary survey was made in which various nitrogenous compounds were tested in different concentrations. The results summarized in Table I indicate the following order of effectiveness for butanediol production: vitamin-free casein hydrolysate > yeast extract > mixtures of amino acids > ammonium sulphate > asparagine > urea, and potassium nitrate.

TABLE I

INFLUENCE OF VARIOUS NITROGEN COMPOUNDS ON STARCH FERMENTATION BY *Bacillus polymyxa* (STRAIN 47)

Nitrogen source (gm./flask)		Butanediol, %	Nitrogen source (gm./flask)		Butanediol, %
Ammonium sulphate	0.16	0.53	Casein hydrolysate	0.2	1.11
	0.32	0.44		0.4	1.32
	0.48	0.52		0.6	1.39
Urea	0.10	0.01	Amino acids*	0.2	0.95
	0.20	0.01		0.4	1.07
	0.30	0.01		0.6	1.11
Potassium nitrate	0.23	0.09	Yeast extract	0.2	—
	0.46	0.06		0.4	1.17
	0.69	0.08		0.6	1.32
Asparagine	0.3	0.16			
	0.6	0.14			
	0.9	0.11			

* *See Appendix for concentrations used.*

This order is somewhat different from that obtained in growth studies, but shows clearly that complex sources of nitrogen are far superior to simple substances. The effectiveness of casein hydrolysate, even that prepared by the same manufacturing concern, was found to vary with the lot used. One lot was definitely inferior to yeast extract for all strains for both growth and diol production; a second (that used in this work), though inferior for growth, was equal to yeast extract for most strains for diol production.

Various combinations of these compounds were then tested as shown in Table II. Evidently the most effective combination is yeast extract with either casein hydrolysate or amino acids, giving a yield of diol of 1.45%, which with 0.55% ethyl alcohol results in a yield of 2.0% total products or 91% of theoretical. Whether the influence of the yeast extract is due to nitrogenous constituents, growth factors, or both is not apparent. Certain strains gave higher yields of diol with yeast extract than with casein hydrolysate, but all produced most efficiently when these two complexes were combined. It will also be noted that yeast extract increased the diol/ethanol ratio to 2.6 to 2.8 as compared with 1.8 to 2.2 for other compounds or combinations of these.

TABLE II

INFLUENCE OF COMBINATIONS OF NITROGENOUS SUBSTANCES ON PRODUCTION OF 2,3-BUTANEDIOL FROM STARCH

Nitrogen source	Butanediol, %	Ethanol, %	Total products, %	Diol / Ethanol
Ammonium sulphate (0.32 gm./flask)	0.45	0.25	0.70	1.8
Casein hydrolysate (0.4 gm./flask)	1.28	0.64	1.92	2.0
Amino acids (0.4 gm./flask)	1.21	0.58	1.79	2.1
Yeast extract (0.4 gm./flask)	1.24	0.46	1.70	2.7
Ammonium sulphate + casein hydrolysate	1.27	0.57	1.84	2.2
Ammonium sulphate + amino acids	1.06	0.50	1.56	2.1
Ammonium sulphate + yeast extract	1.23	0.44	1.67	2.8
Casein hydrolysate + amino acids	1.27	0.59	1.86	2.1
Casein hydrolysate + yeast extract	1.45	0.55	2.00	2.6
Amino acids + yeast extract	1.46	0.54	2.00	2.7

Amino Acid Requirements

The effect of omitting amino acids singly from a mixture of 20 (listed in the appendix) on the fermentation of starch by *B. polymyxa* (strain 47) is clearly shown in Table III. The omission of glycine, isoleucine, methionine,

TABLE III

AMINO ACID REQUIREMENT FOR THE PRODUCTION OF 2,3-BUTANEDIOL BY *Bacillus polymyxa** (STRAIN 47)

Amino acid omitted	Butanediol, %	Amino acid omitted	Butanediol, %
None	1.13	Tryptophane	1.16
Glycine	0.74	Cystine	1.18
Alanine	1.12	Lysine	1.14
Valine	1.12	Histidine	1.13
Leucine	1.13	Arginine	1.11
Aspartic acid	1.25	Proline	1.13
Glutamic acid	1.25	Isoleucine	0.75
Serine	1.23	Methionine	0.84
Phenylalanine	1.10	Asparagine	0.83
Tyrosine	0.98	Cysteine	1.25
		Threonine	1.17

* *Cystine added at rate of 10 mgm./100 ml. medium.*

asparagine (and tyrosine to a slight extent) resulted in depression of diol production. This was confirmed in repeated experiments one of which, along with ethanol analyses, is presented in Table IV. In this and subsequent experiments, cystine, cysteine, and threonine were omitted, and a slightly higher yield was obtained, which was comparable with that given by casein hydrolysate or yeast extract. As will be shown later, this effect was attribut-

TABLE IV

INFLUENCE OF CERTAIN AMINO ACIDS ON PRODUCTION OF 2,3-BUTANEDIOL AND ETHANOL FROM STARCH

Amino acids used	Butanediol, %	Ethanol, %	Total products, %	Diol / Ethanol
1. All except cystine, cysteine, and threonine	1.31*	0.62	1.93	2.10
2. 1—isoleucine	0.87	0.42	1.29	2.10
3. 1—tyrosine	1.19	0.60	1.79	1.97
4. 1—glycine	0.95	0.49	1.44	1.92
5. 1—methionine	0.79	0.39	1.18	2.01
6. 1—asparagine	1.01	0.48	1.49	2.10
7. Isoleucine, tyrosine, glycine, methionine, and asparagine*	1.14	0.55	1.69	2.06
8. 7 doubled	1.20	0.54	1.74	2.22
9. 7 + leucine, tryptophane, arginine, proline, alanine, valine, histidine, lysine	1.32	0.63	1.95	2.10

* *In 7, double concentration normally used.*

able to cystine, which appeared to be inhibitory in certain concentrations. It will be noted from Table IV that five amino acids together (with concentrations doubled or quadrupled to maintain the nitrogen level) gave good yields of diol (1.14 to 1.2%) and that the addition of eight other acids, none of which appeared to be necessary (Table III), resulted in a yield of butanediol (and total products) equal to that obtained with 17 acids. The yield of ethyl alcohol followed closely that of the diol, a ratio of 2 to 2.1 being obtained in most cases.

According to the work of Stokes and Larsen (16) "*Acetobacter suboxydans* can utilize (NH_4)-nitrogen for growth provided it is supplied with the 6 amino acids which it is unable to synthesize or which are formed too slowly for normal development." An attempt was therefore made to determine whether *B. polymyxa* would ferment starch rapidly in the presence of ammonium sulphate provided the five amino acids mentioned above were also present in the medium. It is clear (Table V) that ammonium sulphate is inferior to the five amino acids both at three and five days even though it supplied about four times as much nitrogen as these substances. These two sources of nitrogen combined resulted in a yield of butanediol that was equivalent to the sum of the yields of both components (Treatments 1 and 2) and that was no better at three days than that obtained by doubling the concentration of the five acids. Maximum yields were obtained in three days only on the addition of eight other acids. However, the fermentation will go to completion with the five amino acids alone (with concentrations doubled) if the incubation period is extended to five days (Table V, Treatment 4). This applies as well to conditions in which these acids are individually omitted from a mixture. As the data in Table VI bear out, the marked retardation

TABLE V

COMPARISON OF AMINO ACID MEDIA WITH AND WITHOUT AMMONIUM SULPHATE IN RELATION TO STARCH FERMENTATION

Addenda to basal medium	Incubation (days)	
	3	5
	Diol, %	
1. Ammonium sulphate (0.3%)	0.45	0.86
2. Isoleucine, tyrosine, glycine, methionine, asparagine	0.78	1.16
3. 2 + 1	1.13	1.20
4. 2 (double amounts)	1.19	1.33
5. 4 + 1	1.17	1.31
6. 13 amino acids*	1.34	1.36
7. 6 + 1	1.32	1.33

* *Five above (in 2) + leucine, tryptophane, arginine, proline, alanine, valine, histidine, lysine.*

TABLE VI

EFFECT OF OMITTING CERTAIN AMINO ACIDS ON STARCH FERMENTATION AT THREE AND FIVE DAYS

Acids used	Incubation (days)	
	3	5
	Diol, %	
1. All except cystine, cysteine, threonine	1.43	1.39
2. 1—isoleucine	0.80	1.29
3. 1—methionine	0.72	1.10
4. 1—glycine	0.86	1.31
5. 13 acids*	1.34	1.39

* *See Table V.*

of fermentation after three days, resulting from the omission of isoleucine, glycine, or methionine from the amino acid mixture is largely overcome (except possibly with methionine) after five days' incubation, a fact that was noted earlier in growth studies.

The response of different strains to these amino acids varies, as will be seen in Table VII. Omitting isoleucine and asparagine reduces the yield of diol by all three strains; glycine is required by strain 39, and methionine to some extent; tryptophane and arginine are required by strain 252. Thirteen acids are equal to 17 for strain 39, better for strain 252, and not as effective for strain 82. This last observation was again brought out in a comparison of different sources of nitrogen as shown in Table VIII. The four treatments seem of equal value for strains 39 and 47. Again, with strain 82, 13 acids are

TABLE VII

AMINO ACID REQUIREMENTS OF THREE STRAINS OF *Bacillus polymyxa* FOR PRODUCTION OF 2,3-BUTANEDIOL

Amino acids used	Strain		
	39	82	252
	Diol, %		
1. All except cystine, cysteine, and threonine	0.93	0.95	0.76
2. l−isoleucine	0.78	0.73	0.52
3. l−tyrosine	0.86	0.85	0.73
4. l−glycine	0.57	0.90	0.81
5. l−methionine	0.78	0.86	0.74
6. l−asparagine	0.76	0.61	0.52
7. l−leucine	0.91	0.95	0.72
8. l−tryptophane	0.92	0.98	0.55
9. l−arginine	0.93	0.94	0.62
10. 13 acids*	0.98	0.80	0.95

* *See Table V.*

TABLE VIII

INFLUENCE OF SOURCE OF NITROGEN ON PRODUCTION OF 2,3-BUTANEDIOL FROM STARCH BY DIFFERENT STRAINS OF *Bacillus polymyxa*

Nitrogen source	Strain			
	39	47	82	252
	Diol, %			
Casein hydrolysate	1.04	1.37	0.94	1.01
17 Amino acids	1.09	1.43	0.95	0.67
13 Amino acids	1.04	1.39	0.77	0.99
Yeast extract	1.09	1.36	0.96	1.13

inferior to 17 and with strain 252, superior. Experiments were then carried out to determine the factor responsible for this phenomenon and phenylalanine repeatedly implicated as stimulatory for strain 82 and inhibitory for strain 252. This effect was not demonstrable after five days' incubation.

It was pointed out above that the omission of cystine from the amino acid mixture gave increased yields of 2,3-butanediol. To settle this point an experiment was carried out in which different amounts of cystine were weighed directly into flasks containing casein hydrolysate as a source of nitrogen. The results (after three days' incubation) show (Table IX) that 25 mgm. cystine inhibited all four strains and that 12.5 mgm. was somewhat inhibitory also, especially for strain 82. The four strains tolerated at least 6 mgm. cystine per 100 ml. of medium. This repressive action was not permanent and could not be demonstrated after five days.

TABLE IX

INFLUENCE OF *l*-CYSTINE ON FERMENTATION BY FOUR STRAINS OF *Bacillus polymyxa* GROWING IN A CASEIN-HYDROLYSATE–STARCH MEDIUM

l-Cystine (mgm./100 ml. medium)	Strain			
	39	47	82	252
	Diol, %			
0	0.97	1.21	0.71	0.88
6	0.79	1.22	0.61	0.85
12.5	0.81	1.12	0.44	0.81
25.0	0.64	0.89	0.47	0.61

Discussion

Ledingham *et al.* (9) suggested that it might be preferable to remove the non-fermentable fractions of wheat for the development of an economical 2,3-butanediol fermentation. Ward *et al.* (17) also suggest that the recovery of butanediol would be simpler if starch rather than whole grain were used as the fermentation substrate. The former workers pointed out too that before this could be done "a more comprehensive knowledge of the nutritional requirements of *Aerobacillus polymyxa* is essential." Pure starch with inorganic nutrients was not fermented satisfactorily. Moderately good fermentations resulted from the incorporation of 7.5 to 10% (on weight of mash) wheat germ, shorts, or bran in a pure starch medium but these were impractical quantities. Whole wheat mash itself was found to be improved by yeast extract but not by gluten. These workers concluded that the nitrogen requirements of this organism could be met largely by soluble nitrogenous substances. In further work along these lines Fratkin and Adams (4) also demonstrated the stimulatory effect of bran and shorts in an 8% starch medium on 2,3-butanediol production. Of various other supplements tested, malt sprouts proved to be the most effective, being superior to yeast extract as well.

The use of these complex sources of nitrogen gives no indication of the specific nitrogen requirements of this organism and in fact very little has been published on this aspect of its nutrition. Perhaps the most comprehensive study along this line is that of den Dooren de Jong (1) in which many substances, including amino acids, amines, amides, and urea derivatives, were added singly to different inorganic media with and without glucose. Depending on the conditions, many of these substances could or could not be utilized. It is interesting to note that under conditions favouring the utilization of most of the amino acids no growth was obtained with cystine, which was shown in the present study to be actually inhibitory in certain concentrations. Grossowicz (6) found cystine to be inhibitory for *Neisseria intracellularis*. Snell and Guirard (14) showed that glycine, and to a lesser extent serine and

threonine inhibited their "*Streptococcus lactis R*" culture although this inhibition could be overcome by addition of pyridoxine or alanine to the medium. Niven and Sherman (12) obtained complete inhibition of five enterococci by large amounts of glycine (20 mgm./10 ml.) a phenomenon that was also observed with *Streptococcus lactis* by Niven (11). Greene (5) noted a significant reduction in the growth of *Leptospira canicola* by 10 amino acids including phenylalanine when the amounts used were 0.025% or greater. Fox *et al.* (3) and Fling and Fox (2) demonstrated inhibition of growth and acid production of *Lactobacillus arabinosus* 17–5 by *d*-leucine and *d*-valine but not by their isomers *l*-leucine and *l*-valine. It appears, therefore, that certain amino acids may be inhibitory although the reasons for this effect are obscure as yet.

The results presented in this paper confirm the conclusions of Ledingham *et al.* (9) that soluble nitrogenous materials fulfil the nitrogen requirements of *B. polymyxa*. For most strains, yeast extract, casein hydrolysate, or a mixture of amino acids are interchangeable; however, the first of these exerts an additional stimulating effect when combined with either of the other nitrogen sources, and also possesses the capacity of increasing the diol/ethanol ratio from 2 to 2.6 or more.

The amino acid requirements of strains of *B. polymyxa* vary somewhat, although the need for isoleucine and asparagine seems to be common to the four strains studied. Similar variation of requirements of strains for amino acids has been noted for *Streptococcus salivarius* (13), for *Streptococcus lactis* (11), and for enterococci (12). Furthermore, as Niven (11) and Stokes and Larsen (16) have pointed out, certain amino acids are undoubtedly more important than others; nevertheless satisfactory growth does not occur unless a number of acids, whose individual omission from a mixture does not affect growth, are included in the medium. Whether these acids function merely to raise the nitrogen level of the medium to a certain optimum concentration or whether they function synergistically with other constituents of the medium or both is not understood at present. They may actually be required by the organism, a fact that is not apparent when they are omitted singly from a mixture of 20 acids because of possible transamination reactions (10) or utilization by the organism of closely related amino acids in the mixture. *B. polymyxa* appears to have the capacity to compensate for omissions of important acids by synthesis or utilization of closely related compounds provided it is given sufficient time to do so. The protein still remaining in the starch (0.375% = 15 mgm./100 ml. medium) contributes very little to the nitrogen requirements of the organism.

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APPENDIX

AMINO ACIDS USED (MILLIGRAMS PER LITRE)

Glycine	80	*l*-Cystine	250
dl-Alanine	80	*dl*-Lysine dihydrochloride	400
dl-Valine	320	*l*-Histidine hydrochloride	160
l-Leucine	100	*d*-Arginine hydrochloride	160
l-Aspartic acid	100	*l*-Proline	200
d-Glutamic acid	500	*dl*-Isoleucine	200
dl-Serine	80	*dl*-Methionine	160
dl-β-Phenylalanine	200	*l*-Asparagine	500
l-Tyrosine	140	Cysteine hydrochloride	200
l-Tryptophane	200	*dl*-Threonine	100

ENTYLOMA FUSCUM AND RELATED SMUTS ATTACKING PAPAVERACEAE[1]

By D. B. O. Savile[2]

Abstract

A detailed morphological study has led to the conclusion that *Entyloma Glaucii* should be included in *E. fuscum*. The symptoms of the smut on *Papaver Rhoeas* and *P. somniferum* are described. Study of limited material suggests that the fungus on *Eschscholzia* and *Dendromecon* in California should be retained as *E. Eschscholtziae*. The production of conidia on these hosts is reported for the first time. *Entyloma fuscum* from *Papaver Rhoeas* has been shown to attack *P. atlanticum*, *P. dubium*, *P. glaucum*, *P. monanthum*, *P. orientale*, *P. setigerum*, and *P. somniferum*.

Entyloma fuscum Schroeter

Entyloma fuscum Schroeter, causal fungus of the leaf smut of poppies, has been reported from Europe on *Papaver Argemone*, *P. dubium*, *P. nudicaule*, *P. Rhoeas*, and *P. somniferum*. In addition, the fungus on *Glaucium flavum*, segregated by Dangeard as *E. Glaucii*, and that on *G. corniculatum*, segregated by Ciferri as *E. Fragosi*, are regarded by Liro (4) as belonging to this species. The important European literature references for this smut are listed by Liro and will not be repeated here.

Material recently collected by the writer has led to a study of *Entyloma fuscum*. Inoculation tests have somewhat increased the known host range; and detailed measurements have been made in order to permit a more adequate comparison of the smut on poppy with those on other Papaveraceae. The writer is indebted to Dr. G. L. Zundel, Pennsylvania State College, for specimens of the smut on *Dendromecon* and *Eschscholzia*, and to Prof. H. S. Jackson for the opportunity to examine material in his herbarium and that of the University of Toronto.

Entyloma fuscum has been reported several times in North America, but it has apparently never become firmly established, perhaps because of the scarcity of wild hosts. Its sporadic occurrence suggests that it is carried with the seed in fragments of leaves or capsules. Clinton (2) mentions its occurrence in Maine and New Brunswick, and Gilman and Archer (3) record it from Iowa, all on *Papaver* sp. Zundel (9) records it from Bermuda on *P. Rhoeas* and *P. somniferum*. In addition, Prof. H. S. Jackson has a specimen in his herbarium from Sherwood, N.Y., on *P. somniferum*. It may be noted that Seym. and Earle Econ. Fungi *C* 19 and Reliquiae Farlowianae 693, collected at Kittery Point, Me., in 1889 and 1891, respectively, were issued as on *Papaver* sp., but that both are almost certainly on *P. somniferum*. The writer is obliged to Dr. D. H. Linder for the information that the New

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Brunswick record stems from a specimen in the Farlow Herbarium collected at Campobello in 1898 by Farlow, and that the host appears to be *P. somniferum.*

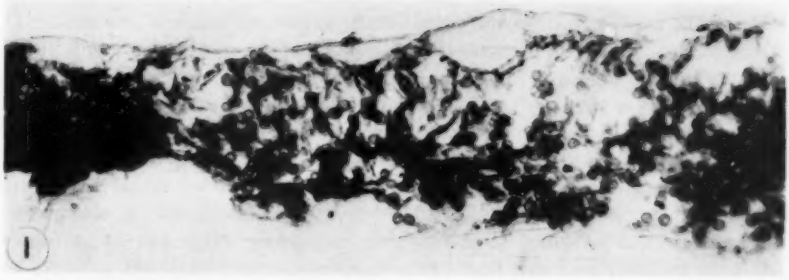
In 1944 this smut was found on *Papaver Rhoeas* in three gardens at Westboro, near Ottawa, Ont., as has already been recorded (5). Further search in 1945 revealed infection of *P. somniferum* in two gardens and of the more commonly grown *P. Rhoeas* in about 10.

Slightly infected *P. orientale* was found in 1944 adjacent to infected plants of *P. Rhoeas.* No conidia were formed on *P. orientale* and the teliospores were somewhat less abundant than in lesions on *P. Rhoeas.* A section through a typical lesion on this host is shown in Fig. 1. It may be mentioned that this photograph, as well as those in Figs. 2, 3, and 5, are from mounts prepared by a rapid sectioning technique devised by the writer (6) in the course of studies of the genus *Entyloma.* By this method sections adequate for such purposes can be secured at a great saving of time. Although there is little difference in the range of spore sizes, the spores in *P. orientale* (Fig. 3) seem to be, on the average, somewhat smaller than in *P. Rhoeas* (Fig. 2). It is doubtful whether the smut, or at least this strain of it, could persist on isolated plants of oriental poppy.

The large, often coalescing lesions on the leaves of *P. somniferum* may seriously disfigure and weaken the plants. On *P. Rhoeas,* of which many named varieties seem to be almost equally susceptible, the leaf injury is generally less severe; under certain conditions, however, extensive lesions may form on the stem, which is often completely girdled when the plant has produced only one or two flowers. This symptom was conspicuous in the hot, dry weather that was experienced in 1944, but was much less frequent in the cooler and moister summer of 1945. Whether it is actually correlated with high temperature or is induced by frequent sprinkling of the plants under such conditions is uncertain. No conidia have been found on these stem lesions, but teliospores are abundant. The teliospores tend to be longer than in the leaves, having been found up to a maximum of 30μ in length; they lie immediately beneath the epidermis, and mounts are conveniently made by stripping off the epidermis with the spores adherent to it (Fig. 4).

A considerable collection of seeds of Papaveraceae had been brought together for planting in the Dominion Arboretum and Botanical Garden in 1945, and the writer was able to utilize some of these species for inoculation with the smut from *P. Rhoeas.* Infection was secured on *P. atlanticum, P. dubium, P. glaucum, P. monanthum, P. setigerum,* and *P. somniferum.* Of these species all but *P. dubium* and *P. somniferum* appear to be new hosts. No infection occurred on *P. Argemone, P. nudicaule, Argemone Barclayana,*

FIGS. 1 to 5. *Entyloma fuscum.* 1. *Section through leaf of Papaver orientale showing teliospore distribution,* × *135.* 2. *Spores in leaf of P. Rhoeas,* × *1180.* 3. *Spores in leaf of P. orientale,* × *1180.* 4. *Spores in stripping from stem lesion on P. Rhoeas,* × *1180.* 5. *Cyst in mesophyll cell of P. somniferum; two spores are seen above,* × *1180.*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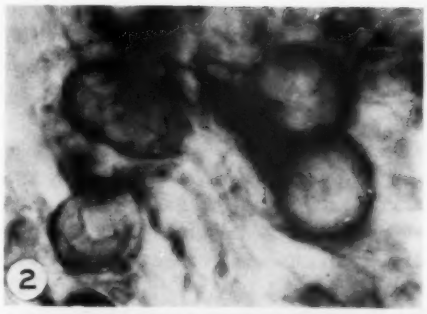
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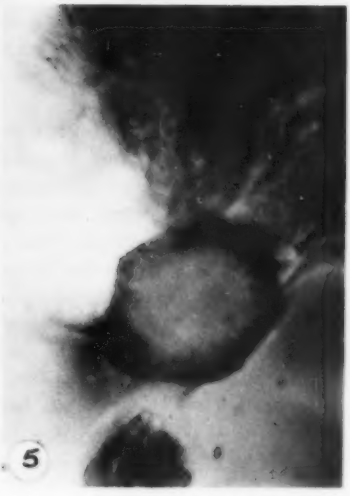
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A. grandiflora, *Celidonium majus*, *Eschscholzia californica*, *E.* sp., *Meconopsis Pratti*, and *Roemeria* sp. The lack of infection on *P. Argemone* and *P. nudicaule* in this test, and of *P. nudicaule* near infected poppies in several gardens at Westboro, suggests that specialization exists in this smut even as it occurs on *Papaver*, for these two species are recorded as hosts in Europe. It is conceivable that some of the species that remained uninfected might prove susceptible under optimum conditions. These tests, although run in summer, were made in the greenhouse in order to minimize the risk of the disease spreading to the main plantings. Temperatures could be kept down only by lowering the light intensity considerably; and this fact, coupled with the tendency of Papaveraceae to be seriously checked by transplanting, was reflected in the finding that no conidia were formed on any plants, and that the teliospores were generally sparser, slightly smaller and thinner walled than is usual. The lesions on *P. somniferum* were, moreover, smaller than typical.

The problem of describing *Entyloma* species adequately for a reasonable segregation on morphological grounds is being discussed in another paper (7). The most useful characters are minimum and maximum length of teliospores, minimum and maximum width of teliospores, range of spore wall thicknesses, density of teliospore distribution, and presence or absence of conidia. The reader is referred to that paper for a fuller discussion of this subject and a consideration of the species concept in *Entyloma*.

Table I gives the essential data for a number of specimens of *Entyloma* on Papaveraceae. Several local collections on *Papaver* have been included, in order to indicate the amount of variation that may occur in a single strain of *E. fuscum*, for without such an indication one cannot assess the distinctness of forms on other host genera. The artificially inoculated material discussed above has been excluded from the table, since it is not regarded as entirely normal. The lesions, although fairly constant on any one host, vary considerably from plant to plant. The lesions are large and round on *Glaucium flavum*, moderately large and round on *Papaver Rhoeas*, and at first round on *P. somniferum*, but soon becoming large, irregular, and sometimes coalescent; on *Dendromecon* and *Eschscholzia* they are small and round. On *Papaver* the conidia are borne hypophyllously in a conspicuous mycelial weft that seems to be derived mainly from the conidiophores but may also result to some extent from the germination of conidia *in situ*. It may be mentioned, in passing, that this weft is a valuable field diagnostic character, for the bacterial leaf spot caused by *Xanthomonas papavericola* also produces round lesions on *P. Rhoeas* and large, irregular ones on *P. somniferum*. On *Glaucium*, *Dendromecon*, and *Eschscholzia* the conidia are borne amphigenously; they are abundant on *Glaucium* and *Eschscholzia*, but are sparse on *Dendromecon*, sections of the latter showing only one to four conidiophores in a stoma.

In Flora Hungarica Exsiccata 603, large spherical cysts, up to 30μ in diameter, occupy many parenchyma cells in the invaded areas of the leaf. In freshly made mounts these bodies are very conspicuous and show pro-

TABLE I

A COMPARISON OF *Entyloma* SPECIES ON VARIOUS SPECIES OF PAPAVERACEAE

Specimen	Host	Teliospore dimensions (μ)			Teliospore distribution	Conidia
		Length	Width	Wall		
Vesterg. Micr. Rar. Sel. 874, *E. Glaucii*	*Glaucium flavum*	9.2–17.0	8.5–14.0	1.0–3.0 (3.5)	3	*A*
DAOM 14369*	*Papaver orientale*	10.0–18.0	8.5–14.0	0.5–2.5	2	—
DAOM 14370	*P. Rhoeas*	9.6–20.0**	8.2–15.9	0.7–3.2	3	*H*
DAOM 15438	*P. Rhoeas*	11.3–16.7	10.8–14.0	0.2–0.7	1	*H*
DAOM 15439	*P. somniferum*	10.5–20.0	9.5–16.0	1.0–4.3	3	*H*
DAOM 15440	*P. somniferum*	11.2–19.5	10.7–16.0	0.7–4.0	3	*H*
Flora Hungarica Exsiccata. 603	*P. somniferum*	10.2–21.5	10.0–15.0	0.7–3.0	3	*H*
Reliquiae Farlowianae, 693	*P. ? somniferum*	10.0–17.0	9.6–15.3	0.7–2.5	3	*H*
Baker Pac. Coast Fungi, 225, *E. Eschscholtziae* (ex. G. L. Zundel)	*Dendromecon* sp.	9.2–18.5	7.5–12.7	< 0.2	4	*A*
Univ. Calif. Herb. 405153, *E. Eschscholtziae* (per G. L. Zundel)	*Eschscholzia californica*	8.7–16.0	7.2–11.5	0.5–1.2 (1.6)	4	*A*

Note: Name given under specimen is that under which it was issued; where no name is given, the specimen was issued as E. fuscum. Under teliospore distribution, 1 = sparse, 2 = moderately abundant, 3 = abundant, 4 = crowded. Under conidia, A = amphigenous, H = hypophyllous, — = absent.

* *DAOM is the identifying symbol of the Mycological Herbarium of the Dominion Department of Agriculture.*

** *In stem lesions spores in this material ran up to 30μ in length.*

nounced radial striations. Some of the material of which they are composed is, however, soluble in lactopherol, and after a few hours the central portion largely disappears and the striations in the outer part become fainter. Fig. 5 shows the appearance of such a cyst after partial dissolution. Similar, but much smaller, cysts, up to 12μ in diameter, were seen in Vestergren's Micromycetes Rariores Selecti 874. In this material the bodies dissolved almost completely soon after mounts were made. Similar cysts have been seen in cells of *Thalictrum dioicum* infected by *Entyloma Thalictri*. These bodies appear to be encysted haustoria, but they differ markedly in composition from those often encountered in the rusts, which, as shown by Thatcher (8), are essentially similar in composition to the spore walls.

The smut on *Glaucium flavum* resembles *Entyloma fuscum* in size, wall thickness, and distribution of teliospores, and differs only in the manner in which the conidia are borne. Liro is unquestionably correct in including this fungus with *E. fuscum*, particularly in view of the close relationship of *Glaucium* and *Papaver*. Admittedly the fungus on *Glaucium* may be specialized upon that host, but probably such specialization is of no higher order than that which is suspected to occur within the smut on *Papaver*. No material of *E. Fragosi* on *G. corniculatum* has been seen, but it seems to have been segregated by Ciferri principally on the basis of the appearance of the lesions

on the two species of *Glaucium*. Without supporting experimental evidence such a separation is entirely untenable. The statement by Ciferri (1), in connection with his study of *E. fuscum* on *Papaver* spp., that the macroscopic appearance is the most important differential character, is invalidated by the finding that a single strain of the fungus produces entirely different lesions on *P. Rhoeas* and *P. somniferum*.

Entyloma Eschscholtziae Harkness

The disposition of the material on *Dendromecon* and *Eschscholzia* is considerably more difficult than of that on *Glaucium*. *Entyloma Eschscholtziae* Hark. was described from material on *Eschscholzia californica* and is apparently confined to California; the fungus on *Dendromecon* was later assigned to it (by Farlow, as the information on the label of Baker Pacific Coast Fungi 225 indicates) with considerable justification in view of the morphological similarity of the fungi, the close relationship of the hosts, and the geographic distribution. Conidia were not noted in the description of this species, and their discovery in these two specimens diminishes the distinction between it and *Entyloma fuscum*. The remaining differences are a slightly smaller spore size, a thinner spore wall than is usual in *Entyloma fuscum* and a greater density of spores in the leaf. If these differences prove to be constant, *Entyloma Eschscholtziae* should probably be maintained. The segregation receives some support from the host relationships and geographic distribution. The fungus on *Dendromecon* has, in the specimen examined, extremely thin spore walls, but this difference alone does not merit specific distinction. It should be noted that *DAOM* 15438, collected in June, 1945, on *P. Rhoeas*, has smaller, sparser, and thinner-walled teliospores, and generally smaller lesions, than the several collections made in July and August, 1944, of which *DAOM* 14370 is typical. The spores measured seem, however, to be mature, and the difference may be due to the specimen having been collected after a long spell of cold, wet weather that may have adversely affected the host. That such a variation can occur emphasizes the risk of basing segregations on minor morphological differences unless such differences have been shown, by the examination of abundant material, to be constant. It is hoped that further collections on *Dendromecon* and *Eschscholzia* will be forthcoming in order that the position of these forms may be better assessed.

Conclusion

For the present, at least, the smut on *Dendromecon* and *Eschscholzia* is to be regarded as a distinct species. The fungus on *Glaucium* should, however, be included with *Entyloma fuscum:* careful measurements of the extremes of spore lengths and widths and of spore wall thickness show no appreciable differences; and in both host genera we find a similar spore distribution pattern and abundant conidia.

Summarizing the data for the various specimens examined, the two species may be described briefly as follows:—

Entyloma fuscum Schroet. Teliospores generally abundant in the host tissue, 9.2−20.0 (21.5) × 8.2−16.0μ (up to 30μ long in stem lesions), wall (0.2) 0.5−3.0 (4.3)μ; conidia amphigenous on *Glaucium*, hypophyllous and generally in a conspicuous mycelial weft on *Papaver*.

Entyloma Eschscholtziae Hark. Spores crowded in host tissue, 8.7−18.5 × 7.2−12.7μ, wall less than 1.5μ; conidia amphigenous, abundant on *Eschscholzia* and sparse on *Dendromecon*.

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BROWN POCKET ROT OF SITKA SPRUCE[1]

By J. E. Bier[2] and Mildred K. Nobles[3]

Abstract

A brown pocket rot of Sitka spruce (*Picea sitchensis* Carr.) occurs in stands on the Queen Charlotte Islands, B.C. The decay, the sporophore associated with it, and the cultural characters of the fungus are described. The causal fungus is regarded as a new species and is described as *Lentinus Kauffmanii* sp. nov. by Dr. Alexander H. Smith, University of Michigan.

In 1942 the attention of the senior author was directed to a decay in Sitka spruce, known locally as "pocket rot," which was encountered in certain areas in the Queen Charlotte Islands frequently enough to make it of concern to the logging companies. The disease was reported to occur in patches, destroying the merchantable contents of many of the largest trees that would otherwise have yielded logs of the superior quality urgently required for aircraft construction. Further, it was stated that there appeared to be no external indications by which the presence of this decay in living trees could be recognized and allowed for in cruises. This resulted in some doubt being cast on the accuracy of the inventories prepared for standing timber, especially in accessible small areas that contained only a limited number of large trees suitable for sawlog production.

A pocket rot in Sitka spruce was described briefly by Mounce (1, p. 22) in the Report of the Dominion Botanist for the year 1926. Specimens of the decay and cultures isolated from it in the Dept. of Agr., Ottawa, Mycological Herbarium and stock culture collection show that it was identical with the rot under investigation. Mounce reported the isolation of the fungus causing this decay from freshly sawn Sitka spruce in British Columbia and also from specimens of Sitka spruce exhibiting "dote," removed from lumber received in England from British Columbia. These cultures remained unidentified until the present study.

Examination of the literature pertaining to the rots of Sitka spruce and correspondence with pathologists in the United States failed to disclose any additional references to a decay of the pocket rot type. Consequently investigations were undertaken to determine the incidence and economic importance of the decay, the causal organism, and, if possible, some criterion by which the disease could be recognized in standing trees.

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The Disease

Geographical Distribution

During the course of this investigation brown pocket rot was found to be generally distributed in the Queen Charlotte Islands. Cultures of the causal fungus have been isolated from infected trees in Alaska, Washington, and Oregon. Dr. Alexander H. Smith has reported in correspondence that sporophores identical with those produced by the fungus causing brown pocket rot have been collected from logs of Sitka spruce in California. It appears, therefore, that the fungus has a wide distribution that probably coincides with the range of Sitka spruce.

The Decay

The decay is a brown pocket rot of the heartwood of living Sitka spruce trees. It may occur as a butt, trunk, or top rot, but it was observed most frequently as a butt and trunk rot confined to the first and second 40-ft. logs, with an occasional infection extending into the third log (Figs. 3, 4). Given sufficient moisture, the fungus may continue to develop in dead trees for long periods. Sporophores of the causal fungus were collected on infected trees that are known to have been felled more than 50 years ago.

In the incipient stage of decay a faint brownish discoloration develops in minute pockets in the wood (Fig. 1). Later the pockets increase in size and become filled with a dark brown crumbly mass that tends to break into cubes, frequently with plates of white mycelium in the cracks. This decayed wood usually drops out of the centre of the pocket leaving large holes edged with rot. The individual pockets are broadly lens-shaped, ranging in size from 12 to 24 in. in length by 4 to 8 in. in width at the middle of the pocket, being elongated in the direction of the grain. They are sharply delimited from the apparently sound wood surrounding them (Figs. 2, 3, 4). In the final stages the pockets may become so numerous that they unite into a continuous column of rot (Fig. 3). It is common to find isolated pockets at some distance from the main body of decay and separated from it by what appears to be sound wood, although it has been shown by cultural methods that the fungus may be present throughout. Because of this distribution of pockets the amount of rot visible on the two ends of a log may be no indication of the extent of internal decay. The difficulty in estimating for brown pocket rot has resulted in scalers culling most logs showing this defect.

Occurrence and Method of Infection

In a general survey of the decays in Sitka spruce in the Queen Charlotte Islands, 1977 trees, from different localities, including both slope and bench

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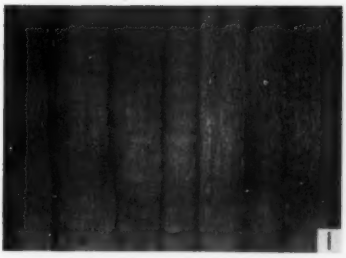
Lentinus Kauffmanii. FIG. 1. *Incipient stage of brown pocket rot.* FIG. 2. *Individual pocket in longitudinal section.* ×*0.125 approx.* FIG. 3. *Brown pocket rot on the stump end of a basal log.* FIG. 4. *Decay 40 ft. along the log from Fig. 3.* FIG. 5. *Brown pocket rot in association with scar.* FIG. 6. *Fruit bodies on Sitka spruce.* ×*0.5 approx.* FIG. 7. *Fruit bodies on the cut surface of an infected log.* FIG. 8. *Fruit bodies on an infected log that had been on the ground for a period exceeding 50 years.* FIG. 9. *Culture on malt agar, six weeks old.*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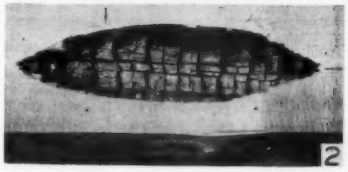
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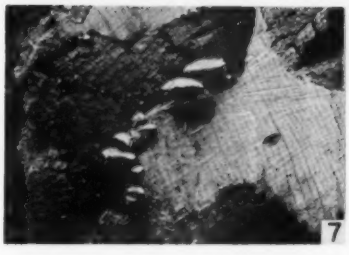
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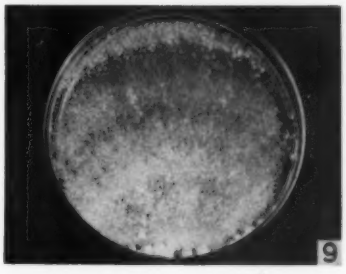
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forest types, were analysed. Brown pocket rot occurred on all the types investigated, being responsible for 91 infections, or 6.4% of the total of 1414 infections in the trees examined. It ranked sixth in the list of decay-producing fungi on the basis of number of infections. The disease could not be regarded as serious in any locality nor was it found to occur solely in patches, causing the total loss of all trees in isolated areas, as had been claimed.

Since the disease frequently occurs as a root and butt rot and was reputed to be destructive in patches, it was thought that infection might be largely through the roots, by contact either with diseased roots or with the causal organism living saprophytically in the soil. However, it was observed that roots served as avenues of entrance for only 56% of the infections, while 36% were associated with scars, 3% with branch stubs, and 5% with dead tops. These latter types of infection, presumably initiated by air-borne spores, resulted in trunk and top rots in isolated trees. This further refutes the popular belief that the disease infects all the trees within a group.

The study failed to demonstrate a relationship between age of the tree and incidence of brown pocket rot. A number of trees in the younger age classes had become infected through scars caused by falling trees (Fig. 5).

Importance

The gross merchantable volume (from stump height to an 11-in. top) of the 1977 trees analysed amounted to 13,968,223 bd. ft. (British Columbia Log Rule*), of which 11.5% was destroyed by decay. Brown pocket rot resulted in a loss of 0.9% of the gross merchantable volume and was the fourth most important decay, following in order of importance conk rot (*Fomes Pini* (Thore) Lloyd), brown butt rot (*Polyporus Schweinitzii* Fr.), and brown crumbly rot (*Fomes pinicola* (Sw.) Cke.).

Since brown pocket rot occurs commonly as a root and butt rot it was of interest to determine its importance in Grade 1 logs, which are cut from the basal region of trees and which have the greatest value for the recovery of clear lumber. The total volume scaled† as Grade 1 in this work amounted to 5,119,267 bd. ft., of which decay had destroyed 7.2%. Brown pocket rot was responsible for the loss of 1.6% of the total volume Grade 1, and was second in importance to brown butt rot (*Polyporus Schweinitzii*) in logs of this grade.

Analyses of trees with basal infections demonstrated that when a large area of rot occurred in the stump end of the basal 40-ft. log, the decay extended into the second log (Figs. 3 and 4). Infections with a small number of isolated pockets at the base usually ran out before the top end of the first log.

No information is available on the persistence of the organism causing brown pocket rot in wood in service. Four cultures submitted for identifica-

* *Province of British Columbia. Log Scale. Victoria. 1944.*

† *The scaling and grading was undertaken in accordance with the standard practice outlined in the following publications:*

Province of British Columbia. Forest Act. Victoria. 1941.

Province of British Columbia. Scaling lessons. Dept. of Lands, Victoria. Revised 1942.

tion that proved to be this species were labelled as having been isolated from "Sitka spruce lumber," one from a mill in Alaska, one from a mill in Oregon, and two from British Columbia. It is probable that they had been obtained from freshly cut lumber. At least two specimens of this decay were encountered in 1925 and 1926 by Dr. Irene Mounce in her study of samples of defective Sitka spruce lumber that had been removed from shipments received in England. In these the pockets were minute, and according to a letter, dated Mar. 18, 1926, from Dr. E. J. Butler of the Imperial Bureau of Mycology, who forwarded the specimens, the defect could not "be detected on the uncut log and only on the finished spars with very close examination". No further records of its occurrence in worked wood are known.

External Indications of Decay

One of the aims of the study was to find some external indication by which the presence of brown pocket rot in standing trees could be recognized. In some species of wood-rotting fungi the fruit bodies are produced on the living trees and these provide a reliable proof of the presence of the decay within the tree. In the case of the organism producing brown pocket rot, the fruit bodies are formed exclusively on infected wood that has been exposed to the atmosphere. Hence they have not been found on the bark of standing trees nor on the ground adjacent to them, and neither fruit bodies nor any other outward symptoms have been observed that would indicate the presence of brown pocket rot in standing trees.

The Fungus

Sporophores

Small agarics were found associated with pocket rot in recently felled trees (Fig. 6), at the margin of pockets of advanced decay on the ends of logs (Fig. 7), and in abundance in the exposed pockets in old logs (Fig. 8). Cultures isolated from the rots and from spores and context of the associated fruit bodies proved to be identical. Hence it was concluded that these were fruit bodies of the organism causing the decay.

Specimens of the sporophores were submitted to Dr. Alexander H. Smith, University of Michigan, for identification. Dr. Smith replied that he had examined previous collections of the fungus and considered it to be an undescribed species, and he kindly consented to describe it for presentation here.

"*Lentinus Kauffmanii* A. H. Smith sp. nov.

"Pileus 3–8 cm. latus, convexus dein late convexus vel planus, saepe lobatus vel ad marginem undulatus, siccus, demum glaber, pallidus dein subincarnato-alutaceous; lamellae confertae, adnatae vel lineato-decurrentes, angustae, pallidae vel sordide pallido-incarnatae, serrulatae; stipes 3–6 cm. longus, 5–12 mm. crassus, aequalis, solidus, centralis vel eccentros, siccus, pallide subincarnato-alutaceous; sporae 5–6 × 2μ; pleurocystidia 60–100 × 7–12μ, subcylindrica vel fusoide ventricosa. Specimen typicum legit A. H. Smith n. 8520, prope Fort Dick, Calif., Nov. 8, 1937; in Herb. Univ. of Mich. et Dept. of Agr., Ottawa, Myc. Herb., conservatum.

"Pileus 3–8 cm. broad, convex, the margin at first incurved, becoming broadly convex to plane and in age the margin frequently elevated, wavy and irregularly lobed, surface dry and at first hoary from a thin cottony covering, soon appearing glabrous and unpolished, whitish from the conspicuous sheen but soon becoming sordid pale incarnate-alutaceous,

when mature the colors varying from pale buff to dull tan with scarcely any pinkish cast visible; flesh tough and cartilaginous, dull pinkish to sordid pinkish buff, odor and taste merely fungoid; lamellae crowded, bluntly adnate but decurrent by long ridges which extend down the stipe for 1–2 cm., narrow to moderately broad, equal, whitish to pale pinkish buff (4), edges finely serrate; stipe 3–6 cm. long, 5–12 mm. thick, equal, solid, central or eccentric, at times nearly lateral, concolorous with the pileus and with the same canescent covering at first, flesh pinkish, base white-felty mycelioid.

"Spores 5–6 × 2μ, subcylindric to slightly curved, smooth, hyaline, not amyloid; basidia 30–34 × 5–6μ, four-spored, pale reddish in iodine; pleurocystidia very abundant, 60–100 × 7–12μ, hyaline, subcylindric to somewhat ventricose, often quite ventricose before elongating; cheilocystidia very abundant, 60–125 × 5–9μ, the apex usually enlarged slightly and the central portion equal; gill trama homogeneous, scarcely colored in iodine, of narrow subparallel to somewhat interwoven hyphae, the hyphae broadest at the cross walls and with abundant clamps; pileus trama homogeneous beneath a turf of more or less upright slender filaments 4–5μ in dia. (these almost form a palisade), no lactifers seen; stipe with numerous hyphae projecting from the surface but not as crowded as over the pileus.

"Scattered to gregarious on spruce logs and stumps along the Pacific Coast from California to the Queen Charlotte Islands. The type was collected at Fort Dick, Calif.

"*Observations:* This species is readily distinguished by the absence of a veil, the very small almost allantoid and non-amyloid spores, conspicuous cystidia, the structure of the cuticle of the pileus and the pale sordid buffy incarnate colors. Kauffman collected it on several occasions in the Pacific Coast area and had considered it undescribed. He found it growing within old logs or in dark places on the under sides of trunks piled up as the result of wind storms. The type, however, was found scattered all over a fallen spruce trunk. Kauffman in his notes compared it with *L. umbilicatus*, but it can be readily distinguished from that species by its lack of an acrid taste, its spores, and the plane to only broadly depressed instead of umbilicate pileus. It is very closely related to *L. adhaerens* (Alb. et Schw.) Fr. but differs in lacking the viscidity of that species, in having smaller spores, and in lacking any appreciable odor and taste. In color, stature, habitat, cystidia and non-amyloid spores it is similar to *L. adhaerens* as described by Konrad et Maublanc."

In this study an abundance of sporophores was found during the months of May, June, September, and October. Invariably they developed from the wood at the margin of pockets that had become exposed to the atmosphere. The stumps, roots, and ground surrounding the bases of infected trees were examined carefully for the presence of fruiting bodies, but in no instance were they found.

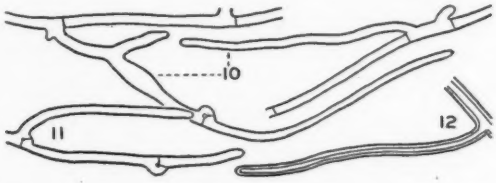
Cultures

In the course of this investigation, 35 isolates of *Lentinus Kauffmanii* have been studied in culture. Of these, four were obtained from either the context or spores of fruit bodies of *L. Kauffmanii* and in each case a culture for comparison was isolated from the accompanying rot. These cultures, whether from rot or associated sporophore, proved to be identical, which established beyond question that *L. Kauffmanii* is the fungus causing brown pocket rot. The remaining cultures were isolated from decays and were identified by comparison with the authentic cultures from fruit bodies.

Each isolate was subjected to the routine procedure followed in the Ottawa Laboratory for the identification of fungus cultures, described by the junior author in earlier publications (2, 3). It was observed that cultures of this species, irrespective of their source, are remarkably uniform and readily recognizable. Furthermore they remain constant, three cultures isolated in 1925 being still identical with the newest isolates. A description of the cultures, based on the 35 isolates referred to above, follows.

Growth Characters

Growth moderately rapid to slow, plates covered in four to five weeks (Fig. 9). Advancing zone even, hyaline, and appressed in zone up to 1 cm. broad, the mycelium so scanty that limit of growth can be determined only by removing lid of Petri dish and examining surface of colony by reflected light. Mat white at first and remaining so or developing pinkish buff (4) to cinnamon buff areas after five to six weeks, appressed, downy to thin woolly, with numerous scattered compact dots or nodules, of which the surface is felty to smooth and skin-like, ranging from 1 mm. or less in diameter on young colonies up to 5–10 mm. on six weeks old cultures. Reverse unchanged. Odour strong, disagreeable. On gallic and tannic acid agars diffusion zones lacking or weak, no growth on either medium.



Lentinus Kauffmanii. FIG. 10. *Hyphae from advancing zone.* FIG. 11. *Regularly nodose-septate aerial hypha.* FIG. 12. *Fibre hypha.* ×600.

Hyphal Characters

Advancing zone: leading hyphae hyaline, thin-walled, with inconspicuous simple septa, frequently branched, the branches soon developing clamp connections, 2.2–4.5 μ diameter (Fig. 10). *Aerial mycelium:* (*a*) nodose-septate hyphae as described above; (*b*) fibre hyphae numerous in older cultures, the walls thick and refractive, lumina narrow or apparently lacking except at tips, occasionally branched, 2.2–3.0 μ diameter (Figs. 11, 12). *Submerged mycelium:* (*a*) nodose-septate hyphae as described above; (*b*) crystals numerous, large, octahedral.

The nodulose surface of the older cultures on Petri dishes and in culture tubes is characteristic and distinct in appearance from any other species in the culture collection. The mycelium of the advancing zone in which hyphae with simple septa give off branches bearing clamp connections, is typical, and is found in the cultures of relatively few species of wood-destroying fungi. Normal sporophores have been produced occasionally on older cultures in tubes, but their formation is too rare to be of diagnostic value. However, even without fruit bodies, it should be possible to recognize cultures of *Lentinus Kauffmanii* readily.

Acknowledgments

The authors wish to thank Dr. Alexander H. Smith for his kindness in examining the fruit bodies and preparing the description of the new species, and Mrs. G. Stewart (Dr. Irene Mounce) for permission to use her notes and photographs.

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COLIFORM CONTAMINATION OF EGGS[1]

By E. H. Garrard[2]

Abstract

Bacteriological examination of 1080 eggs from pullorum infected hens showed 78 (7.2%) to be contaminated with coliform organisms, which were classified into various types. No coliform organisms were isolated from 1000 eggs laid by hens free from pullorum disease.

Introduction

During the past two years an increasing number of coliform isolations have been made at this laboratory from ovaries, oviduct, and liver of hens from pullorum infected flocks. When the opportunity of examining a considerable number of eggs from pullorum infected hens was presented in connection with another project (3), the writer decided to determine whether any of the infected birds laid coliform contaminated eggs. If such contamination were present, it might be a factor in the occasionally reported coliform counts in liquid, frozen, and dried eggs (10, 2, 17).

There is no lack of literature concerning contamination of eggs, but there is disagreement as to how and when organisms enter the egg. In support of the theory that organisms enter the yolk before the shell is laid down, Pernot (18) and Hadley and Caldwell (8) maintained that infection of the normal ovary is possible and Lamson (13) and others have questioned the sterility of the oviduct. Rettger (19) presented the opposite view and found no organisms in 200 samples of normal ovaries examined. According to Maurer (14) who made extensive examinations of both the interior and exterior of eggs from various sources, coliform organisms were absent from the interior of the eggs but were present on the surface of all dirty eggs liberally covered with faeces. It is generally agreed that yolks are more heavily contaminated than egg whites and numerous claims have been made as to the bactericidal properties of the latter (15, 20, 22). Maurer (14), Hadley and Caldwell (8), and Pennington (16) have reported varying degrees of contamination of egg yolk, and Rettger (19) isolated coliform types from yolks on 43 occasions during extensive investigations.

While workers have failed to agree as to whether ovaries may be infected with coliform or other types (other than pathogenic forms), or whether the yolks may become contaminated while passing through the oviduct, it is generally admitted that contamination after laying is responsible for the majority of bacteria within the egg. It has been proved that under certain conditions, organisms can penetrate the shell, and high coliform counts are

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Contribution from Department of Bacteriology, Ontario Agricultural College, Guelph, Ont. This work was assisted in part by a grant from the National Research Council.

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generally the result of handling stored, dirty, and cracked eggs. With the exception of some types of rots such as "sour" eggs, where coliform organisms may be in the majority (9, p. 543) it is doubtful whether they play an important part in egg spoilage. Moreover, Johns (11), analysing coliform counts in powdered egg, indicated that there was little correlation with plate and microscopical counts of viable organisms or the result of sanitary surveys. However, while the coliform group is still regarded as the index of faecal contamination, the presence of *Escherichia coli* and allied types cannot altogether be ignored.

Experimental

Source of Eggs

The eggs from pullorum infected hens were obtained from a flock of 55 hens comprised of Barred Rocks, Light Sussex, New Hampshires, and Leghorns. They were procured from various flocks in Ontario that had previously been tested for pullorum disease by the tube agglutination test. Most of them were either constant or intermittent reactors to both standard and variant (24) pullorum antigens, while a few exhibited reactions of a suspicious or non-specific character. The hens were mixed in several pens in a well ventilated, up-to-date chicken house, situated half a mile from the laboratory. They were tested weekly and the eggs collected daily. Because of lack of facilities, the hens were not trap-nested, nor were the eggs graded. Although an attempt was made to test the eggs as they were received, occasionally a storage period of a few days was necessary.

To compare the results with eggs from non-diseased hens, eggs were also obtained from College flocks in which there were no pullorum reactors. These eggs were ungraded and were treated exactly the same as the eggs from the pullorum infected flock.

Methods of Analysis

As the isolation of types and not numbers was the primary object, only qualitative tests were conducted, nor was there any attempt made to distinguish between possible contamination of yolk and white. The method of analysis followed was somewhat similar to that described by Gibbons, Moore, and Fulton (6). The eggs were inspected for cracks and, if sound, were soaked in a 500 p.p.m. chlorine solution, after which they were rinsed in a 60% alcohol solution and placed in a sterilized, perforated rack to dry. The ends were painted with a strong solution of iodine and the eggs again allowed to dry. Using aseptic precautions, the ends were punctured with a flamed awl, and the contents of the egg were blown, by compressed air blown through sterile glass wool, into a sterile, wide-mouthed flask containing broken glass. Each flask was shaken for at least three minutes after which 1- and 2-ml. amounts of egg meat were transferred in triplicate tubes of lactose brilliant green bile broth. If positive after incubation for 48 hr. at 37° C., transfers were made to eosin methylene blue plates, colonies were picked off after suitable incubation, and the isolates typed according to methods previously described (5).

Results

A total of 1080 eggs from pullorum infected hens was examined during the months of July, August, and September. From 78 eggs, cultures were isolated that produced gas in brilliant green bile broth and that proved, on microscopical examination, to be Gram negative, short rods. By using the classification adopted by the Ministry of Health, Appendix I, Standard Methods (1), which is based upon I. M. Vi. C. reactions, sucrose fermentation, hydrogen sulphide production, and gelatine liquefaction, the cultures proved to be members of the coliform group. The results are shown in Table I.

TABLE I

CHARACTERISTICS OF 78 CULTURES ISOLATED FROM EGGS LAID BY PULLORUM INFECTED HENS

No. of cultures	Type	I. M. Vi. C.	No. of cultures fermenting sucrose	No. of cultures liquefying gelatine	No. of cultures producing H_2S	No. of cultures fermenting lactose at 44° C.
52	*E. coli*, Type I	+ + − −	8	0	0	41
8	*E. coli*, Type II	− + − −	0	0	0	0
0	*A. aerogenes*, Type I	− − + +	0	0	0	0
8	*A. aerogenes*, Type II	+ − + +	8	0	0	0
8	*A. aerogenes*, Intermediate Type I	− + − +	1	0	7	0
2	*A. aerogenes*, Intermediate Type II	+ + − +	1	0	1	0

Because of claims of various workers (4, 21, 23) that fermentation of lactose at 44° C. assists in further separating faecal *Escherichia coli* from allied types, this method was included. It will be noted that approximately 80% of the *E. coli* Type I cultures fermented lactose at that temperature while the *Aerobacter aerogenes* and Intermediate forms showed no sign of fermentation at 44° C.

From these same eggs, in connection with another project (3), 61 cultures of *Salmonella pullorum* were isolated. In only six cases, however, were cultures of *S. pullorum* and coliform organisms isolated from the same egg.

Analysis of 1000 fresh eggs during the months of October, November, and December, obtained from hens negative to pullorum disease, failed to produce a single coliform culture. While it has been claimed that higher counts of organisms are found in eggs during the summer months (13) other work would indicate that such is not the case (18) and it is not thought that the time of year greatly influenced the results with regard to types of coliform organisms isolated.

Discussion

While there is no way of proving that coliform contamination in eggs from pullorum infected flocks was not derived from sources outside of the egg, the negative results from eggs from healthy hens, housed and attended under approximately the same conditions, make it fairly conclusive that the organisms were localized in the hens. It still remains to be definitely proved that coliform organisms readily contaminate the interior of the eggs from infected straw, faecal deposits on the shell, etc., and recent work by Johns and Bérard (12) would indicate that such is not the case.

Because the birds were not trap-nested, it was not possible to determine whether any one hen consistently laid coliform contaminated eggs. Few workers record the isolation of coliform organisms from the ovaries at post-mortem where isolations of *S. pullorum* have been made. When the hens in question were posted, most of the coliform isolations obtained were from those hens that gave suspicious or non-specific reactions to the agglutination test, and from those that yielded *S. pullorum* variant strains from ovaries severely affected. However, the fact that in six eggs both *S. pullorum* and coliform organisms were isolated is an indication that both organisms may be present at times in the ovaries or oviduct and that the pullorum reactor may be responsible for laying coliform contaminated eggs.

Although such contamination may be significant with regard to certain spoilage and in adding coliform counts to egg products, of greater significance to laboratories engaged in annually testing thousands of birds for pullorum disease is the possibility of coliform and allied types of organisms being responsible for suspicious and non-specific reactors. Post-mortem examinations of hens reacting weakly or non-specifically to both the tube and rapid agglutination tests have revealed, on numerous occasions, nothing but coliform types, not only from the ovaries and oviduct but from the liver and spleen. Similar findings have been reported elsewhere (7). It is apparent that a considerable amount of work is necessary before the true significance of such organisms can be determined.

Acknowledgments

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CARBOHYDRATE METABOLISM OF McINTOSH APPLES DURING THEIR DEVELOPMENT ON TREE AND IN COLD STORAGE

PART 1. GENERAL TRENDS[1]

By G. Krotkov[2] and V. Helson[3]

Abstract

For two consecutive years, weekly samples of apples were taken during the development of fruits on the tree and in subsequent cold storage. Following determination of their respiration, the fruits of each sample were analysed for various forms of carbohydrates and also for their insoluble *P* content. The last constituent was assumed to represent the 'protoplasmic factor.' Six different physiological stages are distinguished during the ontogeny of apple fruits. These are based on changes in the direction and in the rates of metabolism of various carbohydrates. The first three stages are passed while apples are on tree; the next one is passed either partly or even completely in storage, depending on the date of picking; the last two are passed entirely in storage. At the beginning of storage there was observed a sharp increase in the sugar content of apples. Since there was no corresponding decrease in the alcohol insoluble residue, it was concluded that such sugars must come from some alcohol soluble substances.

Introduction

It was shown previously (9), that when an apple fruit is placed under standardized conditions and its respiration is followed for a period of time, the kind of respiration record produced will depend on the ontogenetic stage of the fruit in question. In the early stages of fruit ontogeny, respiration records are represented by the preclimacteric periods only. In the middle stages there appears a climacteric rise dividing the whole record into the preclimacteric and postclimacteric periods. In later stages the preclimacteric periods disappear completely, and the records obtained are represented by the postclimacteric periods only. It was also shown that from the beginning of July and onward the bulk of the carbon dioxide was produced in the postclimacteric period, and that the duration of life of a fruit was now directly proportional to the length of this particular period.

It has been suggested that the climacteric rise in respiration might be due to an increase in the concentration of the respiratory substrate. This last increase is brought about by the acceleration of the hydrolytic processes in an apple at this stage of its ontogeny. If this suggestion is correct, it may be assumed that the respiratory rate of a fruit from now on is determined primarily by the concentration of the respiratory substrate, and the duration of its life by the amounts of the reserve substances from which this substrate is produced. Since several forms of carbohydrates are present in an apple

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fruit in large amounts, one might expect that some of these do serve as such reserves. It was decided, therefore, to investigate the carbohydrate metabolism of apples, and this paper presents the results of such investigations.

Materials and Methods

The work was carried out in 1941-42 and 1942-43, using McIntosh apples as experimental material. In 1941-42 all the samples studied came from the same tree, which also supplied all the apples used in the previous work (9). In 1942-43, owing to a series of unfavourable circumstances, the experimental material was not so homogeneous. The first four samples were taken from one tree in a small private orchard near Kingston. All the subsequent samples until the end of December were supplied by another tree in the same orchard. The keeping quality of the apples in 1942-43 was not good, the percentage infected with apple scab was high, and so by the end of December this source of supply was exhausted. A bushel of apples was then purchased from a large commercial orchard about 75 miles from Kingston. This was placed in cold storage, and all the remaining samples were taken from this stock.

Throughout both growing seasons apple samples were taken directly from the tree. In the fall several bushels of apples were picked from the same tree, placed in cold storage at 2° C., and these were the apples that supplied the subsequent samples.

Each year the first sample of apples was taken as soon as the petals fell, which was taken to be the time of the fruit setting. In 1941-42 the first sample taken on May 31st consisted of 341 apples. The next few samples contained progressively smaller numbers of apples, until from July 10th and onward each sample consisted of three apples. In 1942-43 the first sample taken on June 2nd had 334 apples, and after progressive decline in the number of apples in a sample, all the samples taken from July 20th and onward consisted of five to six apples each.

Samples of apples were brought to the laboratory, weighed, and their respiration, expressed as carbon dioxide production, was determined at 2° C. for 24 hr. Determinations of the carbon dioxide production were made by the continuous gas method, using Pettenkoffer tubes as described earlier (9).

After determination of their respiration the apples were frozen at −14° C. Early samples consisting of small apples were frozen *in toto*. Samples of larger apples taken in 1941-42 after July 3rd and in 1942-43 after July 13th were first peeled, cored, quartered, and then frozen. While in a frozen state the apples were passed through a meat grinder, and the pulp so obtained was thoroughly mixed. It was this pulp that was used for the carbohydrate and insoluble *P* analyses.

For the carbohydrate analyses, 12 to 15 gm. of the frozen apple pulp were extracted with 300 cc. of 85% ethyl alcohol under a reflux condenser for 24 hr. The alcoholic filtrate of such an extract was used for the determination of the

sugars, while the alcohol insoluble residue was analysed for starch and hemicelluloses. The alcohol was removed from the alcoholic filtrate under reduced pressure at a temperature below 30° C. The clearing of the sugar extracts, and their decolorization by boiling them with charcoal was done as recommended by Archbold (1). The sugar solutions finally obtained were inverted with hydrochloric acid, using the slow method of inversion (3, p. 373, Sec. 23 (c)). The only change introduced into this method was the extension of the period of hydrolysis from 24 to 48 hr. Hanes' modification (4) of the Hagedorn Jensen method was used for the determination of the reducing power of the extracts. Glucose and fructose were determined in the extracts before inversion, using Widdowson's technique (12), which is based on a combination of the alkaline ferricyanide and hypoiodite oxidation. The only modification introduced into this method was that hypoiodite oxidation was done at 2° C. and not at 1° C. All the results of sugar analyses were expressed as percentage of sugar per fresh weight of apple pulp.

Starch was extracted from the alcohol insoluble residue according to the method described by Hanes (5) by "solubilizing" it with hydrochloric acid in 95% ethyl alcohol while it was being boiled for 12 min. under a reflux condenser. The residue was then filtered off, suspended in distilled water, brought to the boiling point, and the starch was extracted by further digestion for 20 min. in a boiling water-bath. The aqueous extract was made up to volume, an aliquot was inverted with hydrochloric acid (3, p. 373, Sec. 23 (c)) and analysed for reducing power. The results were calculated as glucose and expressed either as percentage of apple pulp, fresh weight, or of the alcohol insoluble residue.

Following extraction of the starch, the alcohol insoluble residue was further digested for three hours in a boiling water-bath with 1% sulphuric acid. The filtrate was freed of sulphuric acid by barium hydroxide, and, after neutralization, its reducing power was determined. The results were calculated as glucose and expressed either as percentage of apple pulp, fresh weight, or of the alcohol insoluble residue.

For the analysis of the insoluble *P* content of apples, 12 to 15 gm. of frozen apple pulp were refluxed for 15 min. in a mixture of 150 cc. of 95% ethyl alcohol and 50 cc. of ether to remove lipids. To extract its water soluble *P*, the apple residue was transferred to a beaker and suspended in 10 times its volume of freshly prepared 5% trichloracetic acid. The suspension was stirred mechanically for 20 min., allowed to stand for two hours, filtered, and dried. The dry residue was placed in an envelope and stored in a refrigerator until the time of analysis.

Incineration of this residue was done in an electric furnace for three hours at 500° C. Phosphorus was determined in the ash according to the method described by King (8), using a Klett–Sommerson photoelectric colorimeter.*

* *The authors wish to express their thanks to the American Academy of Arts and Sciences, which provided the funds necessary to buy the instrument.*

The results were calculated as mgm. of *P* per 100 gm. of apple pulp, fresh weight.

In preliminary work, lipids and water soluble *P* were removed from several samples of apple pulp, and the residue was incubated further with 1% sodium hydroxide at 30° C. The filtrates obtained from such digests gave a negative test for *P*. Apparently apple pulp has no organic phosphorus-containing substances that are insoluble in a mixture of absolute ethyl alcohol and ether, which are precipitated with trichloracetic acid, and from which their phosphorus can easily be split off by mild hydrolysis. The usual substances that answer this description are phosphoproteins. Another phosphorus-containing substance that is precipitated with trichloracetic acid, but from which *P* cannot be released with ease by digestion with 1% sodium hydroxide, is nucleoprotein. The analyses for the insoluble *P* have been carried out, therefore, on the assumption that the amounts of such *P* found in an apple may serve as a measure of the nucleoprotein content. In still more general terms, the insoluble *P* serves as a measure of the 'protoplasmic factor.' While several objections may be raised against such an assumption, it is interesting to examine the data with this in mind.

Results

Figs. 1 and 2 present changes in the respiration and insoluble *P* content of apples in 1941-42 and 1942-43, respectively. Changes in respiration are of the type reported and described earlier (7, 9). In both years and particularly

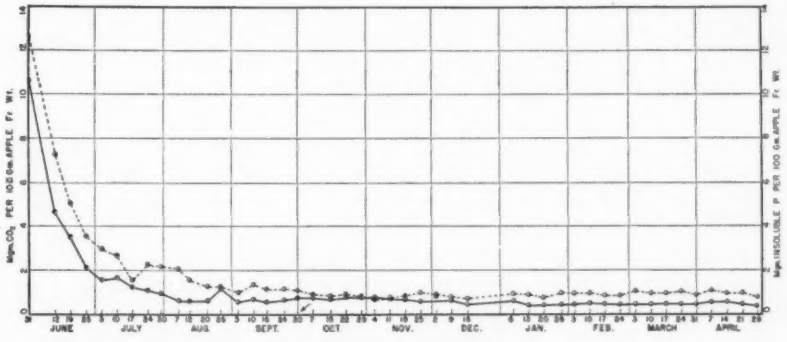

FIG. 1. *Respiration and insoluble P content of apples in 1941-1942.*
◐———◐ *respiration,* ◐ - - - - - ◐ *insoluble P,* ↓ *date of picking.*

in 1942-43 the climacteric rise observed was very small. This is probably due to the fact that respiration was determined at 2° C., and as reported by Smock (11) climacteric is much more evident at high temperature than at low.

The insoluble *P* content of the apples was high initially, and it dropped down very rapidly during the first month of growth. In subsequent months it continued to decline at a progressively slower rate, reaching low values by

the beginning of October in 1941-42, and by the end of August in 1942-43. For the rest of the ontogeny these low values were maintained with slight fluctuations.

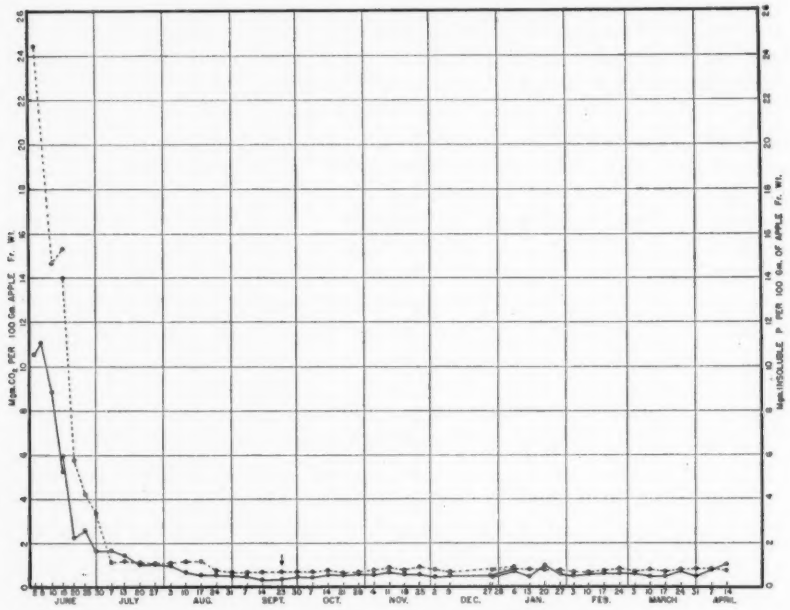

FIG. 2. *Respiration and insoluble P content of apples in 1942-1943.*
◐——◐ *respiration,* ◐ - - - - - - - ◐ *insoluble P,* ↓ *date of picking.*

Close parallelism between the changes in the insoluble *P* content and the respiration of apples supports the original assumption, that the insoluble *P* content of an apple may be taken as a general measure of the 'protoplasmic factor.'

Figs. 3 and 4 give data on changes in various sugars. Examination of these figures reveals that during the development on the tree the total sugar content of an apple was continuously increasing. This increase was at first rapid and then it slowed down. Toward the end of September or early in October there was another rapid rise, reaching the peak of 12.5% in 1941-42 and 10% in 1942-43. After that there was a steady decline for the rest of the storage.

In both years, following a rapid increase within the first two weeks of the season, the glucose content became practically constant for a long time, with a tendency toward a slight increase in the last period of storage. The fructose content was continuously increasing, at first rapidly, then at a slower rate. Having reached a high value, towards the end of October it dropped for a while, and then began a steady rise for the rest of the storage life.

In 1941-42 after more or less steady values for the first two weeks, the invert sugars began to increase at first slowly and then more rapidly. The peak was reached early in October, after which time there came a decline at first

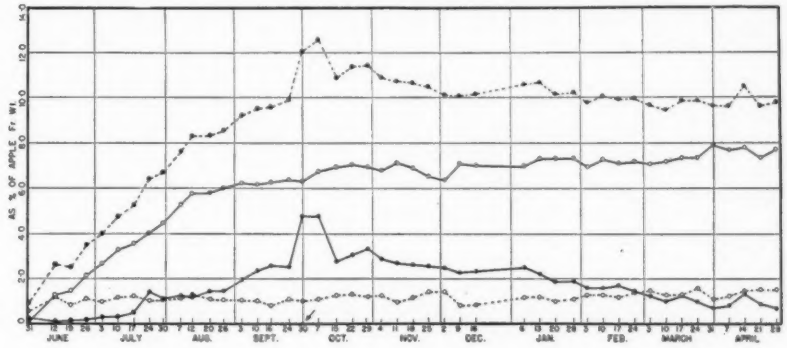

FIG. 3. *Sugar content of apples in 1941-1942.*
○ - - - - - - - - - ○ glucose, ○——○ fructose, ●——● *invert sugars,* ● - - - - - - - - ● *total sugars,* ↓ *date of picking.*

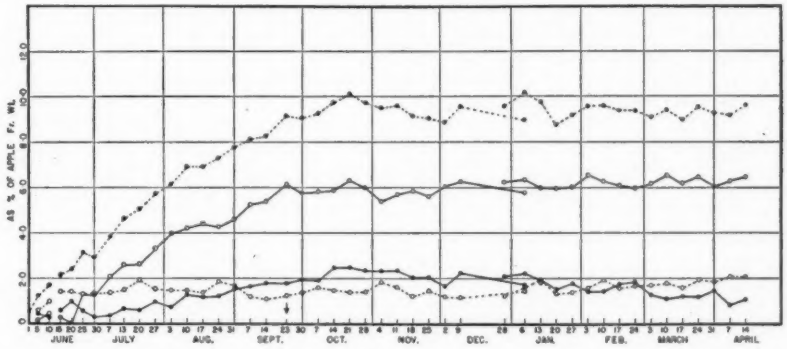

FIG. 4. *Sugar content of apples in 1942-1943.*
○ - - - - - - - - - ○ *glucose,* ○——○ *fructose,* ●——● *invert sugars,* ● - - - - - - - - ● *total sugars,* ↓ *date of picking.*

rapid and then slow throughout the rest of the storage life. In 1942-43 after the initial fluctuations around a low value for about a month, invert sugars began to increase, coming to a peak by the middle of October. Having reached the peak, there followed a continuous, steady decline throughout the rest of the observed life.

Figs. 5 and 6 give data for the seasonal changes in the starch content of apples. Starch was found in apples from the very time of their setting. The amounts present initially were small, and, expressed per fresh weight of an apple, they were declining during the first few weeks. From the middle

of June, however, starch began to accumulate, reaching in 1941-42 a single peak by the first week in August, and in 1942-43 a double peak toward the end of July and by the middle of August. Having reached its peak, it began

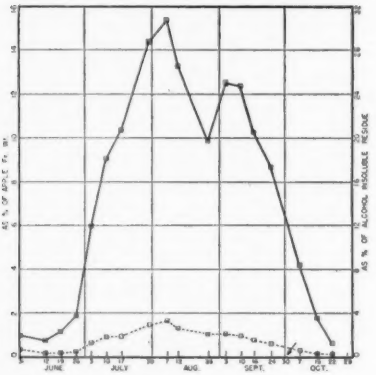

FIG. 5

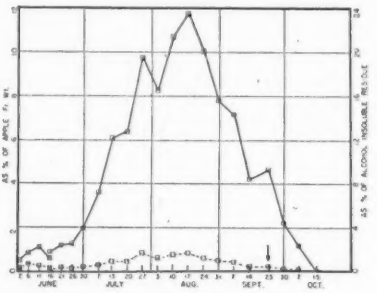

FIG. 6

FIG. 5. *Starch content of apples in 1941-1942.*
□ - - - - - - - □ *as % of the apple fresh weight,* □——————□ *as % of the alcohol insoluble residue,* ↓ *date of picking.*

FIG. 6. *Starch content of apples in 1942-1943.*
□ - - - - - - - □ *as % of the apple fresh weight,* □——————□ *as % of the alcohol insoluble residue,* ↓ *date of picking.*

to disappear, and the last traces of it were gone in 1941-42 by the end of October, and in 1942-43 by the middle of the same month. If starch is expressed as percentage of the alcohol insoluble residue, then its seasonal changes are much more rapid. At its peak, starch formed over 30% of such a residue in 1941-42 and 24% in 1942-43.

Figs. 7 and 8 present changes in the hemicellulose content of apples. In both years early in the season the hemicellulose content, as expressed per apple pulp, fresh weight, was high. It declined rapidly at first, slower later, reaching steady values by the end of October in 1941-42, and by the middle of September in 1942-43. From this time and onward these values were maintained with a very slow and steady decline for the rest of the storage.

If expressed per alcohol insoluble residue, the hemicellulose content appears to be at first sight remarkably constant throughout the whole ontogeny. Fluctuations observed appear to be around an average value of about 8%, which was the same in both years. A careful examination of these fluctuations reveals, however, that their order and timing were very close in both years. Thus in June the percentage of hemicelluloses in the alcohol insoluble residue was steadily increasing, coming to a peak by the end of the month. Throughout July it declined, reaching low values early in August. Then followed another peak, which in 1941-42 was a small one and of short duration, while

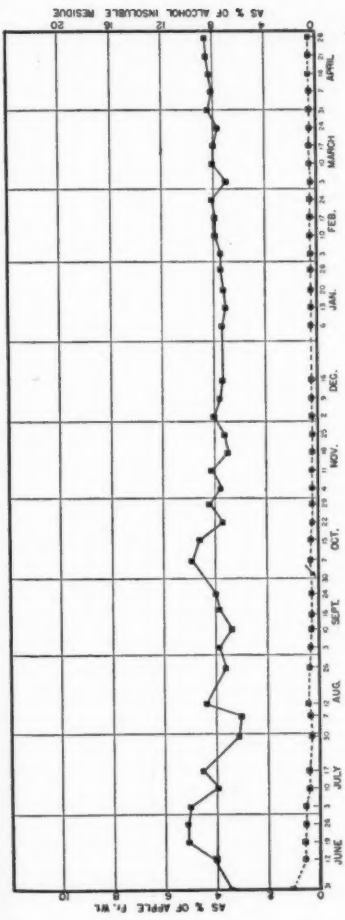

FIG. 7

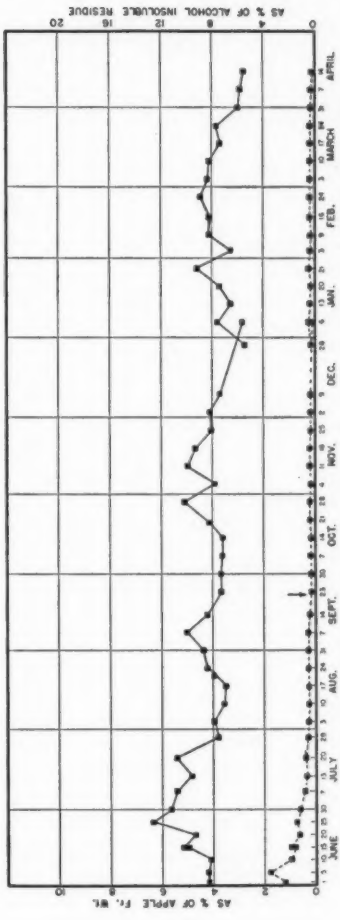

FIG. 8

FIG. 7. *Hemicellulose content of apples in 1941-42.*
■ - - - - - - - - ■ *as % of the apple fresh weight,* ■ ——— ■ *as % of the alcohol insoluble residue.*

FIG. 8. *Hemicellulose content of apples in 1942-43.*
■ - - - - - - - - ■ *as % of the apple fresh weight,* ■ ——— ■ *as % of the alcohol insoluble residue.*

in 1942-43 it was larger and lasted longer. The second largest peak of ontogeny occurred in October, and this was followed by a long and steady decline. From January and onward there was another slow rise.

Discussion

Examination of Figs. 3 to 8 reveals that during its ontogeny an apple fruit passes through a series of successive stages. These are characterized by different concentrations of various carbohydrates as well as by different rates and directions of their metabolism. A transition from one stage to another is indicated by an inflection in a corresponding graph. Six such stages can

TABLE I

THE DURATION AND RATES OF CHANGES IN DIFFERENT CARBOHYDRATES DURING VARIOUS STAGES OF FRUIT ONTOGENY, 1941-42*

—	Total sugars	Glucose	Fructose	Invert sugars	Starch	Hemi-celluloses
Stage 1						
Duration	May 31 to June 12 or 19	May 31 to June 12	May 31 to June 12 or 19	May 31 to June 26	May 31 to June 12	May 31 to June 12
Days	15	12	15	26	12	12
Rate in mgm./day	+108	+52.1	+81.2	−1.5	−15.3	−42.8
Stage 2						
Duration	June 12 or 19 to Aug. 12	Constant	June 12 or 19 to Aug. 12	June 26 to Aug. 12	June 12 to Aug. 7	June 12 to Oct. 22
Days	58		58	47	56	132
Rate in mgm./day	+99		+76.4	+20.8	+26.2	−2.9
Stage 3						
Duration	Aug. 12 to Sept. 24	Constant	Aug. 12 to Sept. 30	Aug. 12 to Sept. 24	Aug. 7 to Oct. 29	
Days	43		49	43	83	
Rate in mgm./day	+38		+10.7	+30.2	−19	
Stage 4						
Duration	Sept. 24 to Oct. 7	Constant	Sept. 30 to Oct. 22	Sept. 24 to Oct. 7		
Days	13		22	13		
Rate in mgm./day	+207		+34.8	+176		
Stage 5						
Duration	Oct. 7 to Dec. 9	Constant	Oct. 22 to Dec. 2	Oct. 7 to Dec. 9		
Days	63		41	63		
Rate in mgm./day	−39.5		−17.9	−39.8		
Stage 6						
Duration	Dec. 9 to Apr. 28	Dec. 9 to Apr. 28	Dec. 2 to Apr. 28	Dec. 9 to Apr. 28		
Days	140	140	147	140		
Rate in mgm./day	−2.4	+3.5	+9.8	−11.8		

** Rates have been calculated from changes in the concentrations of different carbohydrates expressed as percentage of apple pulp, fresh weight.*

be distinguished. The first three are passed while the apple is attached to the tree. The fourth stage is passed either partly or completely in storage, depending on the time of picking. The last two are passed entirely in storage. In 1941-42 the apples were picked after they entered the fourth stage of their ontogeny, and in 1942-43 they were picked before this stage. The duration of each stage and the mean rates of change of the several carbohydrates at each stage are summarized in Tables I and II. Owing to a change in the source of experimental material by the middle of the winter of 1942-43, no comparable data could be computed for the sixth stage of this year.

TABLE II

THE DURATION AND RATES OF CHANGES IN DIFFERENT CARBOHYDRATES DURING VARIOUS STAGES OF FRUIT ONTOGENY, 1942-43*

—	Total sugars	Glucose	Fructose	Invert sugars	Starch	Hemi-celluloses
Stage 1						
Duration	June 1 to June 25 or 30	June 5 to June 15	June 5 to June 25 or 30	June 5 to July 7	June 5 to June 15	June 1 to June 20
Days	27	10	22	32	10	20
Rate in mgm./day	+92.7	+81.3	+49	−2.8	−24.7	−26
Stage 2						
Duration	June 25 or 30 to Aug. 10	Constant	June 25 or 30 to Aug. 24	July 7 to Aug. 24	June 15 to Aug. 17	June 20 to Sept. 14
Days	44		58	48	63	86
Rate in mgm./day	+89.5		+51.6	+18	+11.2	+5.3
Stage 3						
Duration	Aug. 10 to Sept. 30	Constant	Aug. 24 to Sept. 23	Aug. 24 to Oct. 7	Aug. 17 to Oct. 14	
Days	51		30	44	58	
Rate in mgm./day	+41.5		+62.6	+15.2	−13.8	
Stage 4						
Duration	Sept. 30 to Oct. 21	Constant	Sept. 23 to Oct. 21	Oct. 7 to Oct. 21		
Days	21		28	14		
Rate in mgm./day	+49		+5.9	+40.5		
Stage 5						
Duration	Oct. 21 to Dec. 2	Constant	Oct. 21 to Nov. 25	Oct. 21 to Dec. 2		
Days	43		35	42		
Rate in mgm./day	−28.4		−19.8	−19.4		

* *Rates have been calculated from changes in the concentrations of different carbohydrates expressed as percentage of apple pulp, fresh weight.*

The first stage begins at the time of the fruit setting, and it lasts for two or three weeks. During this stage the concentrations of the total sugars are low, although the rate of their increase is the highest for any period of time while the apples are still attached to the tree. It is also the only stage in the life of an apple when the concentration of glucose is higher than that of

fructose. Both of these sugars are rapidly increasing, and by the end of this stage their concentrations become equal. Invert sugars are practically constant. Both starch and hemicelluloses are progressively decreasing, and while concentrations of starch are low, those of hemicelluloses are the highest of all the carbohydrates.

In the second stage the rate at which the total sugars increase remains high, being only slightly lower than that in the first stage. From now, and until the last physiological stage in storage is reached, the glucose content remains practically constant, varying around the value reached at the end of the first stage. Fructose is increasing at practically the same rate as in the first stage. Invert sugars, which up to now have been practically constant, begin to accumulate. The most apparent characteristic of this stage is the rapid increase in the starch content of the apples. In 1941-42 this was taking place at a rate practically twice that of 1942-43.

In the third stage the rate of increase of the total sugars dropped in both years to a value slightly less than half of that in the preceding stage. With the glucose content still constant, this increase was due to a rise in both fructose and invert sugars. There was, however, a fundamental difference in the accumulation of these two sugars in both years. In 1941-42 the main increase was due to invert sugars. The rate of fructose accumulation in this year dropped to a value about one-seventh of that for the preceding stage, while the rate of increase of the invert sugars was up 50%. In 1942-43 the main increase was due to fructose. It is this last sugar that accumulates now at an even greater rate than in the second stage, while the rate for the invert sugars accumulation is somewhat decreased. In both years starch began to disappear. In 1941-42 the rate of its disappearance was lower than that of its accumulation in the previous stage. In 1942-43 these two rates were about the same.

In the fourth stage the rate for the accumulation of total sugars goes up again, and the highest concentrations of the total and invert sugars during the whole ontogeny of an apple occur at the end of this stage. The rate of increase in total sugars, as compared with that for the preceding stage, was increased five times in 1941-42, and only slightly in 1942-43. In both years this increase in total sugars was due mainly to the accumulation of invert sugars and to a lesser extent to fructose.

In the fifth stage there is observed for the first time a decrease in the total sugar content of the apples, and this decrease is shared by both invert sugars and fructose. In 1941-42 the rate of disappearance of invert sugars was about twice that of fructose. In 1942-43 the rates of decrease for both sugars were about equal.

In the last or sixth stage of ontogeny the glucose content, which, in Stages 2, 3, 4, and 5 has been more or less constant, now begins a slow and steady increase. The decrease in total and invert sugars, observed in the preceding stage, is continued. On the other hand, concentrations of fructose, which were declining in the preceding stage, from now on begin to increase.

As is seen from Tables I and II, different stages have various durations even for the same carbohydrate. Thus the first and fourth stages are the shortest, and the sixth is the longest. Neither is the duration of the same stage identical for different carbohydrates. Thus in 1942-43 the length of the first stage varied for various carbohydrates from 10 to 32 days. Variations in the duration of various stages were also observed from one year to another. For all these reasons, taking an apple fruit as a whole, one could give only approximate values for the duration of its successive physiological stages.

While Tables I and II indicate the directions and the rates of changes in the concentrations of various carbohydrates, they do not reveal the magnitude of the changes involved. To get a picture of the absolute amounts of various sugars that were either added or removed from a single apple during any one of these stages, one should take into consideration the rate of changes, the length of the period during which this rate was operative, and the simultaneous increase in the weight of an apple. In order to bring this to light the data obtained have been recalculated as follows. In both years the average weights of single apples were plotted against the time of taking samples, and through these points smooth curves were drawn. The graphs obtained were of the usual sigmoid type. From these graphs it was possible to determine for each year the weight of an average apple at any time of the growing season. Having assumed for each year the average lengths of various stages from the graphs for the total sugars, as summarized in Tables I and II, it was possible to calculate the changes in the fresh weight of a single average apple, as well as in the absolute amounts of various substances that were either added or lost in various stages of ontogeny. The results obtained are given in Tables III, IV, and V.

It is seen from Table III that the final weight of an average apple in 1941-42 was considerably larger than in 1942-43. In both years, however, the greatest proportion of the apple's final weight was produced during the second stage of growth, and it was also in the same stage that the mean daily increment in the weight of an apple was the highest. The third stage, with its mean daily increment only slightly lower, was responsible for practically all the remaining final weight of an apple. Only small additions were made during the first, and when late picking permitted, during the fourth stages. In both years the mean daily increment, expressed as percentage of the initial weight of an apple, was the highest in the first stage, and it was progressively declining. In other words, the relative growth rate of an apple is highest in the first and lowest in the last stages of growth.

In 1941-42 the greatest increase in the total sugar content of an average apple occurred in Stage 2. Somewhat smaller amounts were added during the third stage and approximately 25% during the fourth. In 1942-43 the greatest increase in the total sugars was in the third stage. The next greatest increase was in the second, and finally only 10% was added in the fourth stage. In 1941-42 the mean daily increments for the total sugars were highest in the fourth stage. They were two and a half times higher than those for the

TABLE III

CHANGES IN THE WEIGHT OF AN AVERAGE APPLE

	1941-1942			1942-1943		
	Fresh weight in gm.	As % of the final wt.	As % of the initial wt.	Fresh weight in gm.	As % of the final wt.	As % of the initial wt.
Stage 1	May 31 to June 12 or 19, 15 days			June 1 to June 25 or 30, 27 days		
At the beginning	0.17			0.1		
At the end	3.00			7.0		
Added	2.83	2.2		6.9	7.2	
Mean daily increment	0.189		111	0.255		255
Stage 2	June 12 or 19 to Aug. 12, 58 days			June 25 or 30 to Aug. 10, 44 days		
At the beginning	3.00			7.0		
At the end	80.00			53.0		
Added	77.00	59.4		46.0	48.5	
Mean daily increment	1.330		44.2	1.042		14.8
Stage 3	Aug. 12 to Sept. 24, 43 days			Aug. 10 to Sept. 30, 51 days		
At the beginning	80.00			53.0		
At the end	123.00			95.0	100	
Added	43.00	33		42.0	44.3	
Mean daily increment	1.000		12.5	0.822		1.6
Stage 4	Sept. 24 to Oct. 7, 13 days					
At the beginning	123.00					
At the end	130.00	100				
Added	7.00	5.4				
Mean daily increment	0.538		0.44			

preceding stage. In 1942-43 these increments did not increase in the fourth stage as compared with the third but were decreased by half.

In both years it was during the second stage that the greatest amounts of glucose were added to an apple. In 1941-42 the bulk of fructose was also added in the second stage, being closely followed by the third, and the final 10% was added in the fourth. In 1942-43 the greatest increase in fructose was during the third stage. There was a smaller increase in the second, and the final 10% was added in the fourth stage as before. While in 1941-42 the mean daily increments for the fructose accumulation in the second, third, and fourth stages were about the same, in 1942-43 they varied, and there was a considerable drop in such an increment during the fourth stage.

In 1941-42 the highest amounts of invert sugars were added during the fourth stage and in 1942-43 during the third. In both years the mean daily increments of invert sugars in an apple were continuously increasing from the first to the fourth stages. However, in 1941-42 there was a fivefold increase in this increment during the fourth stage as compared with the third while in 1942-43 the increase was only a slight one.

TABLE IV

INCREMENT PER AVERAGE APPLE AND MEAN RATES OF CHANGE OF VARIOUS CONSTITUENTS PER APPLE PER DAY 1941-1942

—	Total sugars	Glucose	Fructose	Invert sugars	Starch	Hemi-celluloses	Alcohol insoluble residue less starch and hemi-celluloses	Insoluble P
Stage 1	May 31 to June 12 or 19, 15 days							
Added in mgm. per apple	+75	+31	+39	+5	+3	+16	+145	+0.162
As % of the total amount added	0.5	2	0.5	0.1	0.3	4.4	4.8	11.7
Mean daily increment in mgm.	+5	+2	+2.6	+0.36	+0.2	+1.05	+9.6	+0.0108
Stage 2	June 12 or 19 to Aug. 12, 58 days							
Added in mgm. per apple	+6573	+1039	+4585	+949	+996	+310	+2270	+1.05
As % of the total amount added	40	72.9	52.5	15.2	99.7	85.5	75.5	77.5
Mean daily increment in mgm.	+113	+17.9	+79	+16.4	+17.2	+5.32	+39	+0.0180
Stage 3	Aug. 12 to Sept. 24, 43 days							
Added in mgm. per apple	+5550	+220	+3215	+2115	−323	−20	+540	+0.15
As % of the total amount added	34	15.3	36.6	34	32.3	5.5	17.7	10.8
Mean daily increment in mgm.	+129	+5.1	+74	+49	−7.5	−0.46	+12.6	+0.0035
Stage 4	Sept. 24 to Oct. 7, 13 days							
Added in mgm. per apple	+4200	+140	+910	+3150	−369	+56	+60	−0.22
As % of the total amount added	25.5	9.8	10.4	50.7	36.9	15.6	2	15.9
Mean daily increment in mgm.	+322	+10.8	+70	+242	−28.3	+4.32	+4.6	−0.0170
Stage 5	Oct. 7 to Dec. 9, 63 days							
Added in mgm. per apple					−308			
As % of the total amount added					30.8			
Mean daily increment in mgm.					−14.7			

Though in both years the bulk of starch appeared in the second stage, small amounts of it were also synthesized in the first one. From Figs. 5 and 6, presenting changes in the starch content of apples expressed as percentage of the apple, fresh weight, one might draw the conclusion that in the first stage there was hydrolysis and not synthesis of starch. This is due to the disproportion at this stage between the concurrent mean rates of starch production and the increase in weight of an apple. The first rate is low while the second one is relatively high. In 1941-42 the absolute amounts of starch hydrolysed in the third, fourth, and fifth stages were about equal. In 1942-43, 80% of the starch was hydrolysed in the third stage and the remaining 20% in the fourth.

TABLE V

INCREMENT PER AVERAGE APPLE AND MEAN RATES OF CHANGE OF VARIOUS CONSTITUENTS PER APPLE PER DAY 1942-1943

—	Total sugars	Glucose	Fructose	Invert sugars	Starch	Hemi-celluloses	Alcohol insoluble residue less starch and hemi-celluloses	Insoluble *P*
Stage 1	June 1 to June 25 or 30, 27 days							
Added in mgm. per apple	+209	+93	+86	+30	+12	+47	+335	+0.241
As % of the total amount added	2.2	7.1	1.4	1.3	2.9	27.4	17	37.7
Mean daily increment in mgm.	+9.5	+4.2	+3.9	+1.36	+0.55	+1.7	+12.4	+0.0089
Stage 2	June 25 or 30 to Aug. 10, 44 days							
Added in mgm. per apple	+3490	+692	+2150	+648	+406	+91	+1053	+0.335
As % of the total amount added	36.2	53.2	35.8	27.7	97.1	52.9	54.5	53.2
Mean daily increment in mgm.	+79.2	+15.7	+48.8	+14.7	+9.2	+2.1	+23.8	+0.0076
Stage 3	Aug. 10 to Sept. 30, 51 days							
Added in mgm. per apple	+4930	+515	+3253	+1162	−333	+5	+320	+0.058
As % of the total amount added	51.3	39.7	54	49.6	80	2.9	16.3	9.1
Mean daily increment in mgm.	+96.5	+10	+63.7	+22.8	−6.5	+0.1	+6.3	+0.0014
Stage 4	Sept. 30 to Oct. 21, 21 days							
Added in mgm. per apple	+990	−10	+510	+500	−84.5	+29	+240	
As % of the total amount added	10.3	0.7	8.8	21.4	20	16.8	12.2	
Mean daily increment in mgm.	+47	−0.5	+24.3	+23.7	−6.5	+1.39	+11.4	

In both years the bulk of hemicelluloses was formed in the second and to some extent in the first stages. Little change took place during the third stage, and a further slight increase was observed in the fourth. Similar observations on the accumulation of hemicelluloses in developing apples were made by Widdowson (13).

The greatest amounts of the alcohol insoluble residue less starch and hemicelluloses were added in the second stage, with progressively smaller additions during the third and fourth stages.

In 1941-42 the apples were picked after the beginning of the climacteric rise in sugars that is characteristic of the fourth stage. One might claim, therefore, that such a rise was due to an increased importation from the tree. In 1942-43 such a rise occurred after picking, and one has therefore to rule this explanation out. A rise in the sugar content of apples after picking has been reported by other workers also (1; 6, pp. 106-124; 10, pp. 70-75). We can conclude, therefore, that such a rise is not due to an importation from the tree but to the appearance of sugars from some substances already present in the apple at the time of picking.

Table VI presents changes in the total carbohydrate content of apples during different stages of storage. During the fourth stage of fruit ontogeny there is a considerable rise in the sugar content of apples, which in 1941-42 was more than twice that in 1942-43.

TABLE VI

CHANGES IN THE TOTAL CARBOHYDRATE CONTENT OF APPLES DURING DIFFERENT STAGES OF STORAGE*

	1941-42	1942-43
Stage 4		
Total carbohydrate content (total sugars, starch, hemicelluloses) at the beginning	10728	9312
Total carbohydrate content at the end	13134	10286
An addition of	2406	974
CO_2 produced during this period, expressed as glucose	163	168
Total	2569	1142
Stage 5		
Total carbohydrate content at the beginning	13134	10286
Total carbohydrate content at the end	10289	9043
A loss of	2845	1243
CO_2 produced during this period, expressed as glucose	631	380
Difference	2214	863
Stage 6		
Total carbohydrate content at the beginning	10289	
Total carbohydrate content at the end	9801	
A loss of	488	
CO_2 produced during this period, expressed as glucose	925	
Difference	437	

* *Expressed as mgm. of total carbohydrates per 100 gm. of apple, fresh weight. Owing to a change in the source of apples by the middle of the winter in 1942-43, data for the sixth stage in this year are omitted.*

As seen from Tables IV and V, starch, hydrolysed during this stage, could account only for less than 10% of these sugars, and there was a simultaneous slight synthesis and not hydrolysis of hemicelluloses. The alcohol soluble residue less starch and hemicelluloses could not be the ultimate source of these sugars, since it was shown in the same tables that the amounts of such a residue were still increasing during this stage. This last observation is supported by other workers (1, 13).

One is forced, therefore, to conclude that the bulk of these sugars must come from some alcohol soluble substances, and three types of such substances may be tentatively suggested. First, they are substances that are precipitated from the extracts during clearing with lead. Second, they escape hydrolysis under the mild conditions adopted in this work. Third, these sugars are produced from some non-sugar substances.

The disappearance of sugars from apples during the fifth stage is two to three times in excess of their utilization in respiration. This suggests their transformation into some other forms that again escape analysis. Archbold and Barter (2) observed an excess of 17 to 30% in the amounts of carbon disappearing from stored apples in the form of total sugar and acids above amounts lost as carbon dioxide. In the sixth stage the disappearing sugars have contributed approximately one-half of the respiratory substrate, while the other half must have come from some other source.

The absolute amounts of sugars released during the fourth stage are considerable. In 1941-42 and 1942-43 they formed 25 and 10% of all the sugars observed in apples at the peak of their sugar content. They are represented by a mixture of invert sugars and fructose, with sometimes one and sometimes the other predominating.

Equally large amounts of sugars, also represented by a mixture of both invert sugars and fructose, disappear without any traces during the fifth stage. The amounts involved in such transformations are too large to be omitted. Elucidation of the nature of these substances, which give rise to sugars in the fourth stage and into which sugars are transformed in the fifth stage, is necessary for the understanding of the carbohydrate metabolism of apples.

Progressive changes observed during the ontogeny of apples can be summarized as follows. In the first stage of ontogeny, which begins at the time of petals falling, high and rapidly declining rates of respiration, coupled with the equally rapidly decreasing concentrations of the insoluble *P*, suggest a progressive decrease in the relative amounts of living matter. Expressed as percentage of the apple, fresh weight, the hemicellulose content declines very rapidly, while that of starch only slightly. Concentrations of total sugars rapidly go up, and such sugars are represented mainly by fructose and glucose. Invert sugars remain either stationary or even decline somewhat. The relative growth rate of the fruit is the highest of its whole ontogeny, though as a result of its small weight the absolute amounts of various substances added during this stage are very small.

In the second stage respiration and concentrations of the insoluble *P* decline further, though at a slower rate. Concentrations of total sugars expressed as percentage of the apple, fresh weight, are increasing at only a slightly lower rate than in the preceding stage. This time the increase is due mainly to fructose and to a lesser extent to invert sugars, while the glucose content remains constant. Starch concentrations are increasing, while those of hemicelluloses decline further, though at a much slower rate than in the preceding stage. The mean daily increment in the fresh weight of an apple is the highest for the whole ontogeny and close to one-half of the final weight of an apple is formed during this stage. The absolute amounts of various constituents added during this stage are high. The bulk of hemicelluloses, glucose, insoluble *P*, alcohol insoluble residue less starch and hemicelluloses, and practically all of the starch accumulate in this stage.

In the third stage respiration reaches its lowest value, and then it begins to go up in the climacteric rise. Concentrations of the insoluble *P* reach low steady values, which are maintained for the rest of the ontogeny. The rates of increase in the concentration of the total sugars drop to one-half of those for the preceding stage. This increase may be due mainly either to invert sugars, and in such a case an increase in fructose is low, or to fructose, and then the rate of increase for the invert sugars is low Hydrolysis of starch is probably the most striking feature of this stage. The absolute increase in the weight of an apple, though less than in the preceding stage, is still considerable, and for this reason the absolute amounts of various sugars added are large. The alcohol insoluble residue less starch and hemicelluloses is still being added, though in small amounts, and the accumulation of insoluble *P* in an apple may be completed.

A climacteric rise in respiration observed to begin by the end of the preceding stage is now followed by a rise of sugars in the fourth stage. There is an increase in both invert sugars and fructose. Sometimes it is one sugar that accumulates to a greater extent and sometimes the other. Since small increases in the alcohol insoluble residue less starch and hemicelluloses, and also in the hemicelluloses themselves, are still observed during this stage, and since the starch hydrolysed could contribute less than 10% of the increase of total sugars, such a rise must be due to an appearance of sugars from some alcohol soluble substances. Respiration still continues to rise. Hydrolysis of starch, begun in the previous stage, may be completed. If this stage begins before the fruits have been picked, a slight increase in the weight of an apple may still occur.

In the fifth stage respiration reaches its peak and begins to decline. Total sugars begin to decrease, their fall being shared by both invert sugars and fructose. During this stage more sugars disappear from an apple than could be accounted for by respiration.

In the sixth stage a further decrease in the total sugars now occurs at the expense of invert sugars only. Fructose begins to accumulate again, and glucose, which for the four preceding stages was practically constant, begins a steady rise. The amounts of carbon dioxide produced in respiration are now considerably in excess of the amounts of sugars simultaneously lost from apples.

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